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THE SPECIFICITY OF SIALYLTRANSFERASES IN PLASMA MEMBRANE ENRICHED PREPARATIONS FROM EMBRYONIC CHICKEN LIVER CELLS

by

BRAD K. BENDIAK

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled "The specificity of sialyl-transferases in plasma membrane enriched preparations from embryonic chicken liver cells," submitted by Brad K. Bendiak in partial fulfilment of the requirements for the degree of Master of Science in Cell Biology.

ABSTRACT

Two β-galactoside:sialyltransferases have been found to be present in plasma membrane preparations of 13-day embryonic chicken livers. One of these transfers N-acetylneuraminic acid (NAN) to galβ1→3galNAcα-R structures, and another transfers this sugar to galβ1→4glcNAcβ-R structures, and possibly to galβ1→4glc (lactose). The latter sialyltransferase has a pH optimum of 5.5, a temperature optimum of 30°C, and half-saturating values of 180 μM and 17 μM, respectively, for galactoside termini of desialyzed α_1 acid glycoprotein, and cytidine-5'-monophospho-N-acetylneuraminic acid. The enzyme was activated about ten-fold in 1% Triton X-100, and was inhibited by CTP, CDP, CMP, and Ca^{++}. Plasma membranes were characterized by their morphology in electron micrographs, and the relative purification of Mg^{++}-dependent CTPase and Mg^{++}-dependent ATPase in the preparation (as observed by Sanford and Rosenberg, 1972). Characterization of the linkage of NAN to the acceptor was attempted, but has not yielded conclusive results as yet. The importance of glycosyltransferase specificity is discussed, with special regard to the mechanism of biosynthesis of glycoconjugates, and the possible role played by cell surface glycosyltransferases in intercellular phenomena.

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INTRODUCTION

Complex glycoconjugates at the cell surface are ideal candidates as informational molecules. They are, conformationally, relatively stable molecules, and are capable of being linked together to produce a great variety of stereoisomers (natural glycoconjugates have been reviewed by Spiro, 1973; Montreuil, 1975; and Kornfeld and Kornfeld, 1976). Alteration of either a single anomeric configuration or a linkage of a single sugar in a glycoconjugate would result in a molecule which could display, in a biological sense, entirely different informational properties.

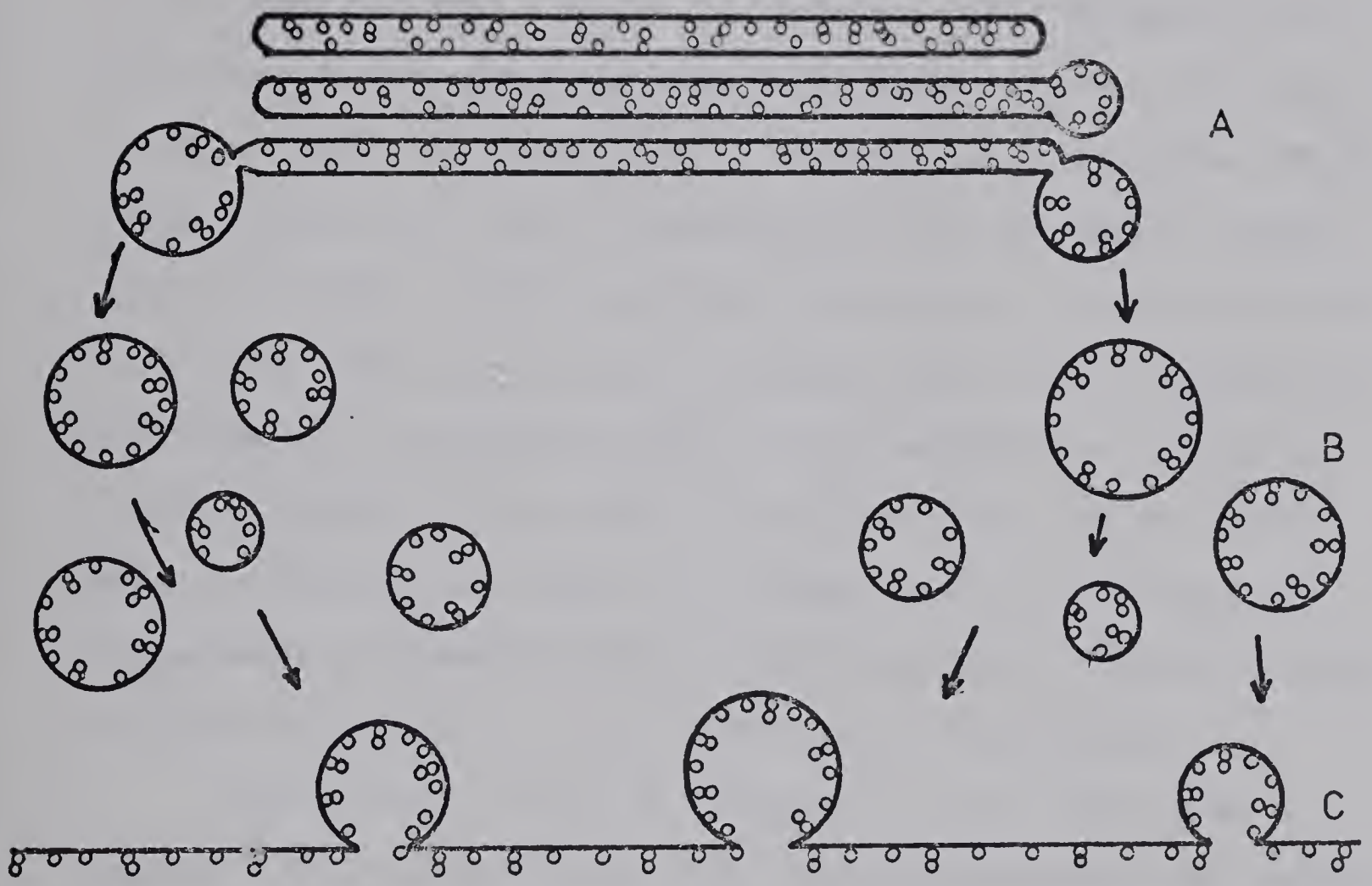
The glycosyltransferases are a group of enzymes which are involved in the synthesis of complex carbohydrates. They catalyze, in a very precise manner, the transfer of specific carbohydrates from sugar donor molecules, which may be either a nucleotide-sugar or a poly-isoprenoic-phosphate-sugar intermediate, to other acceptor carbohydrate structures (reviewed by Schachter and Rodén, 1973; and Waechter and Lennarz, 1976). Recent investigations (Carne and Watkins, 1977; Paulson et al., 1977a; Prieels and Beyer, 1979) have shown that several different glycosyltransferases are specific for at least the terminal disaccharide structure of the acceptor molecule. The linkage between the sugars in the terminal disaccharide of the acceptor is of importance, because different linkages or anomeric configurations in disaccharide isomers can occur which may not be recognized by the active site of a particular transferase.

The synthesis of complex polysaccharides involves the input of a large amount of genetic information. Whereas a protein is the product of a single gene, a glycoprotein is the result of the action of several genes, those coding for the glycosyltransferases, as well as those which code for the structures of the enzymes which synthesize the sugar-nucleotide and sugar-polyisoprenoid intermediates. In short, synthesis of glycoconjugates involves the precise positioning of sugars in a stereospecific fashion to create a complex molecule which may, at the cell surface, store information important in cellular or intercellular processes.

In eukaryotic cells, peripheral sugars are added to glycoproteins inside the flattened vesicles of the Golgi apparatus. The glycosyl-transferases in this organelle are presumed to be oriented toward the interior of the vesicles (Figure 1). Vesicles derived from the Golgi apparatus are believed to fuse with the plasma membrane of the cell, and the glycoproteins and possibly the glycosyltransferases present in the vesicles become externally located in the plasma membrane. This process has been supported by a variety of studies (Jamieson and Palade, 1971; Tartakoff et al., 1974; Schramm, 1967; Schachter and Rodén, 1973; and Whaley et al., 1975).

It is known that particular cell types within embryos are capable of identifying each other and as a consequence adhere in a selective manner. Specific cellular affinities appear in the embryo during gastrulation (Townes and Holtfreter, 1955) and are crucial for the correct organization of the body plan of the early embryo. The ability of the cells to recognize one another becomes more selective as cells follow diverse pathways of differentiation into distinct tissues with

Figure 1. The cellular mechanism of externalization of complex carbohydrates. Synthesized glycoconjugates leave the maturing face of the Golgi apparatus (A) in small vesicles (B) which bud off the flattened Golgi sacs. These vesicles fuse to the plasma membrane (C), thereby externalizing the vesicular contents, among them carbohydrate structures synthesized in the Golgi apparatus.

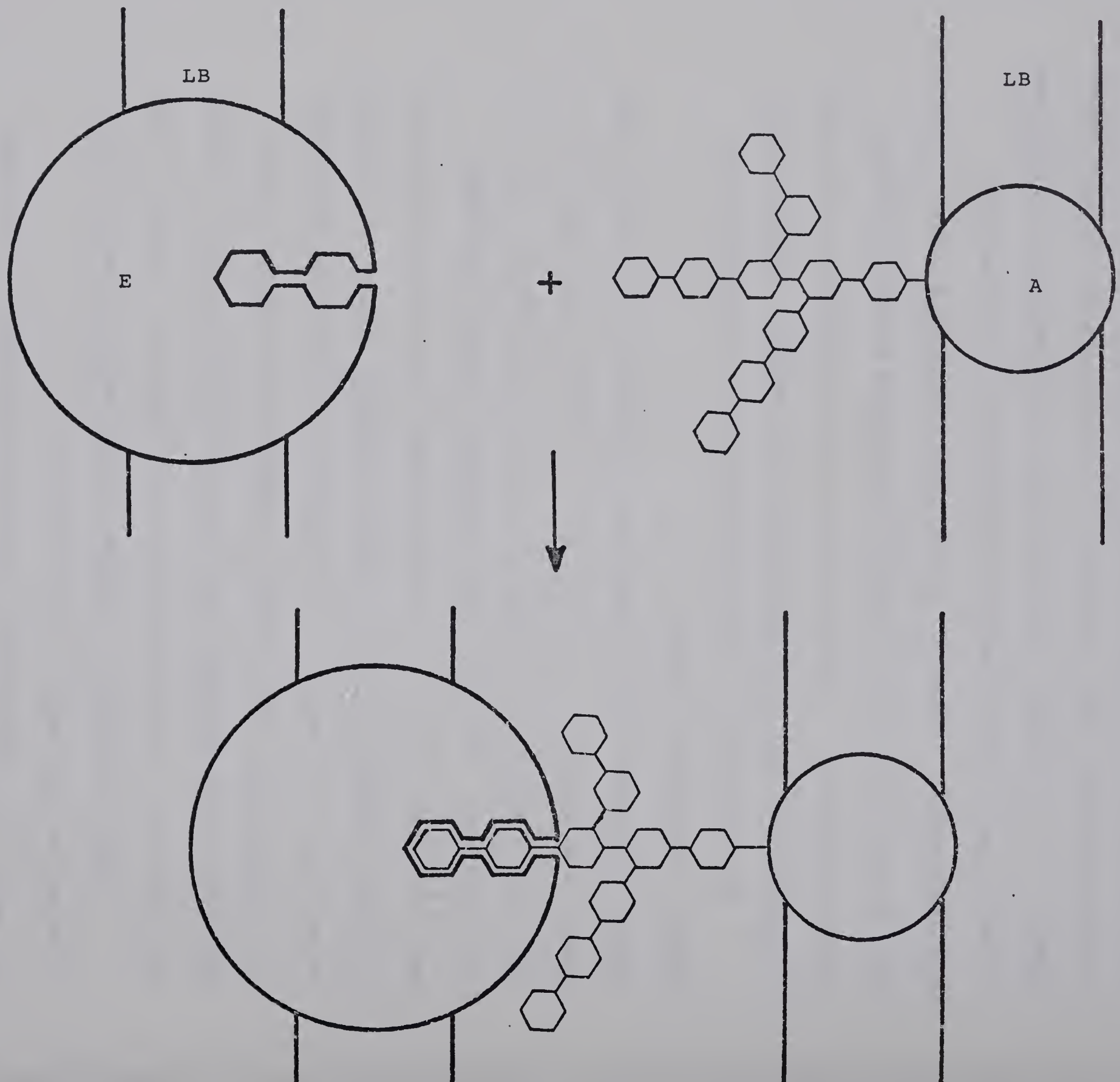

A
B
C

specialized functions.

When cell suspensions obtained from different embryonic tissues such as liver and retina are combined and allowed to aggregate under suitable culture conditions, they will first adhere to one another to form a mixed aggregate. Within the aggregate, cells belonging to the same tissue will coalesce with each other forming distinct cell groups (Moscona, 1964). This phenomenon of "sorting out" demonstrates that the cells, in some way, carry the information for the assembly of tissues within the embryo. There is evidence that supports the notion that the information which allows cells to recognize each other is present at the cell surface. As mentioned before, complex carbohydrate structures, which are present asymmetrically in the outer surface of the plasma membrane (Nicolson and Singer, 1974; Hirano et al., 1972) have been suggested as the informational molecules which may be involved in intercellular recognition (Cook and Stoddart, 1973; Sharon, 1975).

Roseman (1970), and Roth, McGuire and Roseman (1971) have suggested that the specificity of the glycosyltransferases might explain why particular cells specifically adhere to cells of the same type, as observed by Townes and Holtfreter (1955). They reasoned that in the absence of a sugar donor molecule, a glycosyltransferase could bind the specific carbohydrate structure that it utilizes as an acceptor. If a transferase was located on the cell surface, it could bind specific saccharide structures on adjacent cell surfaces. A number of glycosyltransferase-acceptor bonds could result in adhesion between two cells; the adhesion could be very specific, depending upon the particular glycosyltransferase present (Figure 2). The presence of extracellular

Figure 2. The hypothesis of Roseman (1970). Formation of the enzyme-substrate complex between adjacent cell surfaces. The lipid bilayer is labelled as LB, the enzyme as E, and the glycoprotein acceptor as A.


LB
E
+
LB
A

sugar donor was postulated by Roth et al. (1971) to break the intercellular bonds, as the transferase glycosylated its specific acceptor. The cells could then separate, now possessing a modified saccharide structure in their surface glycoconjugates.

Since the proposal of Roseman (1970), many cell surface glycosyltransferases have been reported to be present in a variety of cells (reviewed by Shur and Roth, 1975). Many reports, however, are subject to criticism since they are based on indirect experiments in which intact cells are incubated in a solution containing sugar-nucleotides. As Keenan and Morré (1975) and Deppert and Walter (1978) have stated, many reports may be invalid, because apparent cell surface activity can be artifactual. Cell lysis, with subsequent release of internal glycosyltransferases, or degradation of nucleotide-sugars at the cell surface with subsequent internalization of sugars, could yield the results which have been interpreted as indicating the presence of cell surface transferase activity.

The purification of glycosyltransferases in preparations of purified plasma membranes would provide much stronger evidence for the actual localization of these enzymes at the cell surface. It is likely that in the intact cell the glycosyltransferases in plasma membranes could be oriented so that the active site is externally located, due to the way in which Golgi membranes are thought to be externalized (Figure 1). Conclusive evidence for the presence of glycosyltransferases at the cell surface could be provided if it could be demonstrated that a large molecular weight acceptor, incapable of entry into the cells, and specific for a particular transferase, is glycosylated when present in

the medium surrounding the cells. Provided proper controls were run for any cell lysis that may occur, such a procedure should indicate the orientation of a particular glycosyltransferase in the cell membrane.

Investigators have partially purified glycosyltransferases in plasma membranes from several different cell systems. In 1968, Hagopian, Bosmann and Eylar purified, over 140-fold, a collagen: glucosyltransferase in plasma membranes of HeLa cells. These investigators also reported that two other enzymes which transfer galactosyl, and N-acetylgalactosaminyl groups were present in the cell fractions containing "smooth internal" membranes, and were purified about 50-fold. These enzymes were not found in the plasma membrane fraction. In 1971, Barber and Jamieson isolated plasma membranes from platelets and showed that the membranes contained a collagen:glucosyltransferase that was purified 19-fold. These authors postulated that the enzyme could result in adhesion of platelets to collagen fibres, resulting in hemostasis. Bosmann (1972) also characterized a sialyltransferase present in platelet plasma membranes. This enzyme was concentrated 54-fold in a plasma membrane preparation. He suggested that this enzyme might be responsible for platelet aggregation, since inhibitors of platelet-platelet aggregation (Uridine diphosphate (UDP), Adenosine diphosphate (ADP) and aspirin) also inhibited sialyltransferase activity.

Lloyd and Cook (1974) purified plasma membranes from 16C malignant rat dermal fibroblasts and found them to contain a galactosyltransferase and a sialyltransferase. These authors found that removal of N-acetylneuraminic acid (sialic acid, NAN) from the surface of intact cells resulted in increased cell aggregation. They suggested that

removal of sialic acid residues resulted in exposure of galactoside groups on cell-surface glycoconjugates. This latter sugar might function as an acceptor for a sialyl or galactosyltransferase, resulting in the formation of intercellular bonds.

Pricer and Ashwell (1971) first reported that membrane receptors in rabbit liver cells are involved in the recognition of certain glycoproteins and their subsequent removal from the circulation. In the particular case of ceruloplasmin, a copper-carrying glycoprotein, the removal of terminal sialic acid and exposure of galactose residues in this molecule is necessary for its recognition by the membrane receptors of the liver cell (Ashwell and Morell, 1974). It was found that the presence of sialic acid on the liver cell membrane was also necessary for this clearance, and purified membranes from this tissue have been found to contain sialyltransferase activity. Recent studies carried out by Ashwell's group (Paulson et al., 1977c) show that the purified membrane receptor is a lectin which contains carbohydrate residues covalently attached to the protein of the molecule. Removal of sialic acid from the carbohydrates of the lectin molecule permits the lectin to bind to its own galactoside residues. Re-sialylation of the lectin's carbohydrates allows the lectin to interact with the galactosides of circulating glycoproteins. Other authors (Aronson et al., 1973) have suggested that a galactosyltransferase, also present in the plasma membrane of the liver cell, may be the actual receptor that binds the asialoglycoprotein.

Kim et al. (1971) reported the presence of a sialyltransferase in preparations of human erythrocyte ghosts. Since erythrocytes contain

no internal membranes, contamination with the latter can be ruled out in these preparations. Podolsky et al. (1974) purified rabbit erythrocyte ghosts and discovered the presence of a galactosyltransferase. These investigators showed that the galactosyltransferase itself was a glycoprotein, since the purified enzyme was precipitable with concanavalin A, a lectin which most effectively binds α-mannosides.

Parrodi and Martin-Barrientos (1977) detected enzymatic activity in rabbit reticulocyte plasma membranes involved in the synthesis of polyisoprenoid-bound mannose and polyisoprenoid-bound N-acetylglucosamine. This membrane preparation was also capable of transferring the above-mentioned sugars to endogenous acceptors.

In 1975, McLean and Bosmann showed that purified plasma membranes of the alga *Chlamydomonas moewusii* contained cell-surface sialyl-transferase activity. When membranes from the gametes of two different mating strains (+) and (-) were mixed, enhanced transfer of NAN to endogenous acceptors was observed. These authors suggested that a sialyltransferase-acceptor complex was formed during the mating reaction which was responsible for the adhesion of mating strains.

Merritt et al. (1977) have purified plasma membranes from rat livers, and have shown that galactose, N-acetylglucosamine, mannose, and glucose could be transferred to endogenous acceptors in the plasma membrane fraction. No NAN could be transferred from cytidine-5'-monophosphate N-acetylneuraminic acid (CMP-NAN) to endogenous acceptors.

In many of the above-mentioned studies, glycosyltransferase activity was tested by the transfer of sugars to endogenous acceptors present in the particular preparations. The study of individual glycosyltransferases is difficult when only endogenous acceptors are

used, since the latter generally are present in subsaturating amounts. For the quantitation of enzyme activity, as well as the examination of the specificity of glycosyltransferases present in membrane preparations, the use of defined exogenous acceptors present in saturating amounts is required. Ideally, each glycosyltransferase should be purified to homogeneity, so that it could be studied independently of other enzymes. However, this is not always feasible, especially in those cases where, in some system of biological interest, the transferase is present in small quantities. If a glycosyltransferase is not purified, it is of importance that the acceptor which is used contain only one type of "potential acceptor site" for this enzyme. For instance, fetuin, a glycoprotein from fetal calf serum, when desialyzed, has been used as an acceptor for sialyltransferases in a number of different reports. Desialyzed fetuin contains two different types of acceptor sites, a terminal galβ1→4glcNAc and a terminal galβ1→3galNAc (Spiro and Bhoyroo, 1974; and Baenziger and Fiete, 1979; see Figure 6). Each of these galactoside termini could potentially serve as an acceptor for a different sialyltransferase, making interpretations of kinetics, specific activities, and other enzyme characteristics rather difficult. Other glycoproteins, such as bovine and ovine submaxillary mucins, when desialyzed, have also been commonly used as acceptor molecules in sialyltransferase studies, yet these molecules contain several potential acceptor sites (Baig and Aminoff, 1972; Bertolini and Pigman, 1970; and Carlson, 1968).

One acceptor which is likely to prove useful for sialyltransferase studies is human α_1 acid glycoprotein, a glycoprotein present in human

serum. This protein, when desialyzed, contains galactose linked only β1→4 to glcNAc residues (Figure 6). Another glycoprotein of interest is the "antifreeze glycoprotein" of Antarctic fish (Shier et al., 1975), which contains only the disaccharide galβ1→3galNAc linked to the protein core. These glycoproteins are not, however, commercially available.

The importance of using acceptors with only one kind of "acceptor site" is that competition studies can be performed using impure preparations of glycosyltransferases, and the enzymes can be sorted out according to their ability to glycosylate specific acceptors. Since purification of transferases is difficult in many systems, the use of defined acceptors is imperative in order to obtain reliable information about the above-mentioned enzymes.

Another approach that has recently proven useful for the study of the possible cellular localization of glycosyltransferases is electron microscopy autoradiography. Bernacki and Porter (1978) have shown that in L1210 leukemic cells, ^{3}H-labelled NAN was transferred from CMP-^{3}H-NAN to the extracellular surface, but was not found to an appreciable extent in the Golgi apparatus. Electron micrographs showed a large number of silver grains at the cell surface (84%), with less than 1% present within the Golgi apparatus. On the other hand, UDP-^{3}H-galactose appeared to be degraded to galactose in the extracellular medium, since an incubation of cells with UDP-^{3}H-galactose resulted in the Golgi apparatus being heavily labelled (43%), the remaining label being dispersed in the cell. This type of study yields fairly convincing evidence for the locale of a glycosyltransferase. These authors, however, found no transfer of sialic acid to exogenous

acceptors in the medium. They also did not run controls for enzyme which may have leaked from cells into the surrounding medium. This work is important because degradation of CMP-NAN at the cell surface with subsequent internalization of NAN could be ruled out by these experiments.

Another convincing approach was that used by Yogeeswaren et al. (1974), in which glycosphingolipid acceptors were covalently linked to glass beads which were not able to be phagocytosed. When intact hamster NIL or BHK cells and exogenous sugar-nucleotides were incubated with the beads, the glass-bound acceptors were glycosylated. The extracellular location of the enzyme would be necessary if such glycosylation were to take place. However, controls for leakage of enzyme into the medium were not performed.

After providing positive evidence for the presence of a particular glycosyltransferase at the cell surface, the obvious question which arises is "What is its role there?" if indeed it has a role. Is it involved in completing synthesis of carbohydrate units? Is it simply a residual enzyme which is externalized onto the surfaces, coincidentally with the glycoproteins present in the vesicles derived from the Golgi apparatus? Could the enzymes be involved in cell-cell recognition, as Roth et al. (1971) have suggested? Could they function along with cell-surface glycosidases in altering certain cell-surface heterosaccharides important in intercellular processes? Determining these biological functions pose investigative problems which have not yet been examined to a great extent. It is important, in attempting to determine a biological role for a cell-surface glycosyltransferase that the specificity of the enzyme be examined. For instance, if the

transferase was involved in cell-cell adhesion, then specific inhibition of the enzyme with its particular competitive saccharide substrate could prevent the adhesion. Likewise, if the enzyme was important in altering surface components at certain developmental periods, the morphogenetic effect resulting from specific inhibition could prove interesting. Even if glycosyltransferases are simply carried out onto the cell surface from the Golgi apparatus as a normal process of enzyme turnover, a study of their specificity would yield insight into the mechanism of synthesis of complex polysaccharides within the Golgi apparatus.

In embryonic systems, tissues are often undergoing rapid growth, and numerous cellular interactions occur. Unique synthetic enzymes, such as glycosyltransferases, might be discovered more readily in such systems. Den et al. (1975) have partially purified seven different glycosyltransferases in particulate fractions of embryonic chicken brain. Arnold et al. (1973, 1976), using intact cell suspensions of embryonic chicken liver found that mannose, galactose, N-acetylglucosamine, L-fucose, and N-acetylneuraminic acid could be transferred to endogenous acceptors from their respective nucleotide-sugar intermediates. These authors provided some evidence indicating that this transfer occurs at the cell surface, but failed to run controls for cell lysis. It is possible that the transferase activities seen by these authors actually reflect the presence of glycosyltransferases in the extracellular medium, due to cell lysis. Nevertheless, Arnold et al. showed that embryonic chicken liver appeared to be a good source of glycosyltransferases. Between 10 and 13 days of incubation, considerable synthesis is likely occurring, because the livers nearly double in size daily (personal

observations). In addition, Dalton (1934) reported that the Golgi apparatus becomes more extensive in the cells of the developing liver between 7 and 14 days of incubation.

Previous work had shown that cells of the differentiated liver agglutinate in the presence of *Ricinus communis* agglutinin, a lectin which binds specifically β-D-galactoside groups (Moscona, 1971; Zalik and Cook, 1976). This indicated that glycoconjugates displaying this terminal sugar were present at the surfaces of these embryonic cells. In addition, an endogenous lectin which specifically binds β-D-galactoside groups has been reported to occur in the developing chick liver (Kobiler and Barondes, 1977; Cook et al., 1979). It is possible that the presence of terminal galactoside groups on membrane receptors may also be regulated by the addition of terminal sialic acid by a sialyltransferase. It is also possible that cell-surface glycosyltransferases are directly involved in liver cell adhesion, by the scheme hypothesized by Roseman (1970). Since enough liver tissue can be obtained for biochemical investigations, and since plasma membranes have been prepared from this tissue by two different procedures (Sanford and Rosenberg, 1972; Zalik and Cook, 1976), the investigation of glycosyltransferases present in plasma membranes was initiated. From the indications in this report, the embryonic liver would appear to be a good system in which to study these enzymes.

The mechanism of glycoconjugate synthesis has received increasing attention in the past decade. However, very few of the glycosyltransferases have as yet been fully characterized. The reasons for this are worth mentioning, since an understanding of how cells synthesize at

once a number of glycoconjugates will stem from studies of the individual transferases involved.

The first major obstacle is the availability of substrates. Some of the nucleotide-sugar intermediates, first synthesized by Roseman et al. (1961), are now commercially available, and are also available radioactively labelled. CMP-NAN, however, presents a problem, since it has not been chemically synthesized. It is prepared enzymatically, and is only commercially available in micromolar quantities and containing a radioactive label. Indeed, controversy still exists as to whether the compound is the α or the β anomer (Comb et al., 1966; Stone and Kolodney, 1971). Polyisoprenoic-sugar intermediates, such as dolichol phosphate mannose, must be purified from biological systems, or chemically synthesized by the individual researcher, since these intermediates are also not commercially available.

Obtaining specific sugar acceptors is probably the major difficulty in glycosyltransferase studies. Because the transferases often require specific disaccharide substrates, the presence of particular enzymes can go completely unnoticed unless an appropriate acceptor is present. Although certain glycoproteins may contain the particular disaccharide within their structure, the preparation of defined disaccharides from larger units can be difficult. Narasimhan et al. (1977) attempted to obtain defined acceptors for a glcNAc-transferase, by sequential treatment of glycoproteins with glycosidases; difficulty arose as a result of incomplete action of the enzymes, as well as the inherent microheterogeneity in the carbohydrate structures present in the glycoproteins.

Chemical synthesis, in many cases, may be the best way of obtaining defined saccharide acceptors in large quantities. Using defined and homogenous acceptors is imperative in glycosyltransferase assays. The use of undefined endogenous acceptors, or exogenous acceptors with different potential acceptor sites, makes interpretation of the numbers and types of transferases in biological preparations very difficult. Also, purification of various glycosyltransferases requires a large number of assays to be performed, which would require the use of large amounts of substrate; many substrates are currently not available commercially.

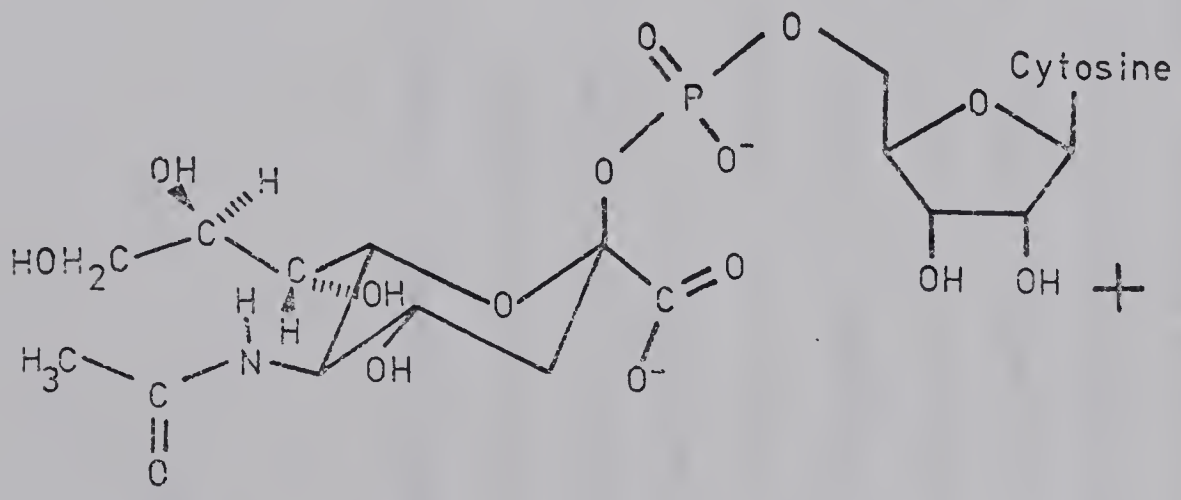
A second problem involves the assays which detect specific glycosyltransferases. Large molecular weight acceptors can be routinely assayed by precipitation of the labelled product with trichloroacetic acid (TCA) and phosphotungstic acid (PTA), or by separation on Sephadex G-50 or G-75. Glycolipids can usually be extracted with organic solvents. However, when disaccharide or monosaccharide acceptors are used, it is necessary to separate the product, usually isotopically labelled, from the labelled nucleotide-sugar donor, as well as from the free labelled sugar which may occasionally appear due to the breakdown of the sugar donor during incubation. Chromatography on anion-exchange columns should separate nucleotide-sugars from the product, but separation from the free sugar is not always routinely possible. The procedure devised in this report permits routine separation of small sialosides from both sialic acid and CMP-NAN, and should be useful in assaying for various sialyltransferases. Routine separation of neutral sugars from assay products, if necessary, might be achieved by rapid,

high resolution procedures such as high pressure liquid chromatography. The product should, if possible, be kept in a small enough volume to permit counting (usually less than 5 ml), especially if counts are low.

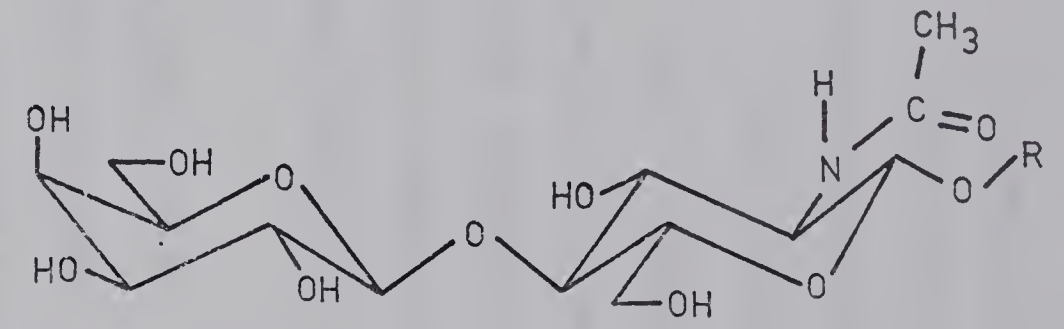
A third obstacle in studying glycosyltransferase catalysis is determining the nature of the product. In many systems, the amount of available enzyme is limited, and the product is synthesized in picomole, or nanomole quantities. Due to this, nuclear magnetic resonance spectroscopy, or conventional chemical procedures such as permethylation, or degradative oxidations, cannot be used. The sensitivity of chemical procedures can be increased by specifically labelling the acceptor monosaccharide with a radioactive isotope, such as ^{3}H or ^{14}C. Examination of the labelled products obtained could make linkage determination possible, using much smaller quantities of product. Such a procedure has already been performed by Van den Eijnden et al. (1977), using permethylation procedures.

In this report, two sialyltransferases have been shown to be present in plasma membrane preparations of embryonic chicken liver. One of these was characterized and found to catalyze the reaction shown in Figure 3. This enzyme was specific for βgal 1→4glcNAc structures, and possibly βgal 1→4glc. A second sialyltransferase added NAN to a terminal βgal 1→3αgalNAc structure. These enzymes, both present in the same membrane preparation, did not compete with one another for acceptors. In the same solution, each enzyme could independently add sialic acid to its specific disaccharide acceptor site.

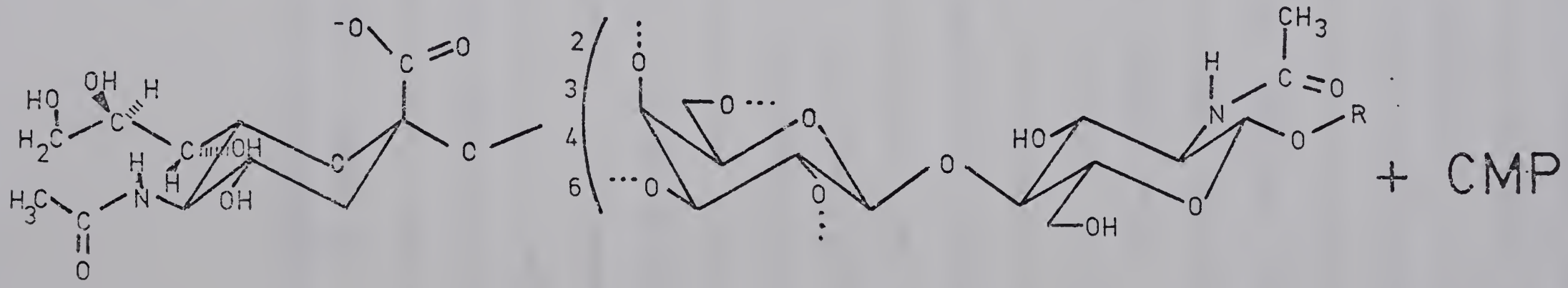
Figure 3. Reaction catalyzed by the sialyltransferase present in the plasma membranes of liver cells from 13-day chicken embryos.



CMP-NAN
(drawn β)



βgal 1→4βglcNAc 1→R



α Sialoside

+ CMP

MATERIALS AND METHODS

Plasma Membrane Preparation

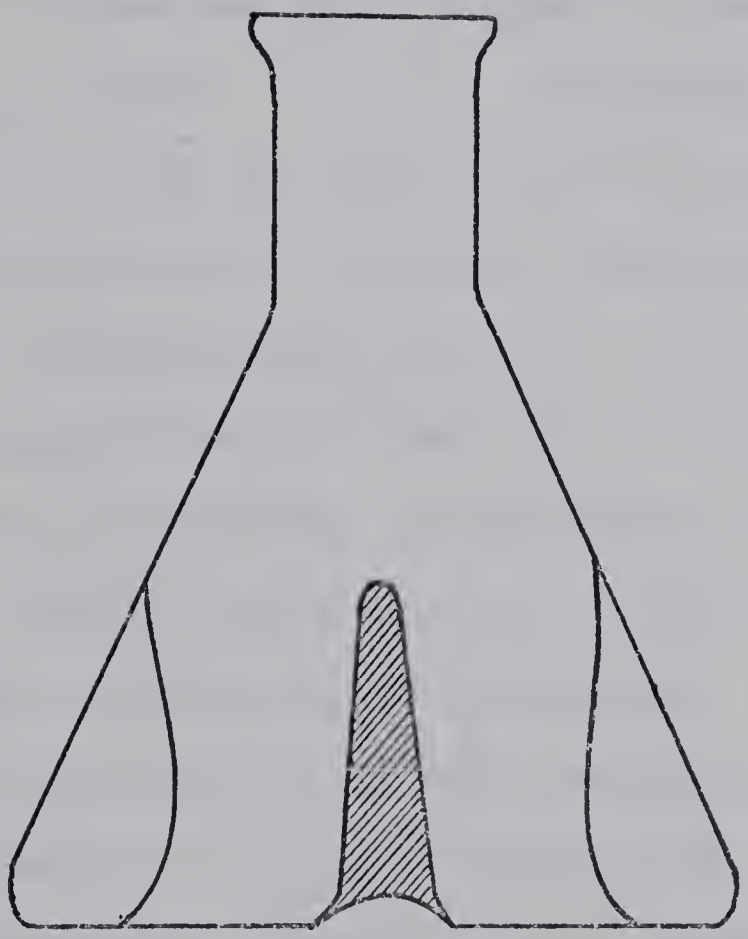
All chemicals were obtained commercially, and were of analytical grade. White Leghorn chicken embryos were used in all experiments. Sixty fertile eggs, obtained from the University of Alberta farm, were incubated for 13 days at 37° C, with a relative humidity of approximately 65%. Generally, after 13 days of incubation, fertility was greater than 95%. Embryos were removed, and prior to excision from the embryos, the livers were perfused with calcium-magnesium-free (CMF) Tyrode's solution (136 mM NaCl, 2.7 mM KCl, 0.32 mM $NaH_2PO_4 \cdot 2H_2O$, 12 mM $NaHCO_3$, 5.5 mM glucose, pH 7.5). Gall bladders were removed, and the livers were placed in 20 ml of ice-cold CMF Tyrode's solution. Tissues remained in this solution for up to 2 hours until all livers were removed from embryos. The CMF Tyrode's solution was then decanted and the livers were minced with scissors.

Plasma membranes were prepared according to Zalik and Cook (1976). This procedure results in the release of large membranous sheets, or "ghosts" from cells. The use of sodium borate in hypotonic medium causes cells to release their plasma membranes; most of the remaining cellular constituents gel, and can be separated from the plasma membranes. The isolation procedure was performed as follows. Approximately 20 ml of ice-cold CMF Tyrode's solution containing 2 mM disodium (ethylenedinitrilo) tetraacetate (EDTA), pH 7.2 was added to the minced livers, and initial cell dissociation was begun by pipetting

with a large bore Pasteur pipette (inner diameter of 3-4 mm). After three or four one-minute periods of pipetting, the suspension was placed on ice for about 10 minutes. It was then pipetted with a flamed Pasteur pipette (inner diameter of 0.5-1.0 mm) for approximately 5-10 minutes, until no visible clumps of tissue remained. The cell suspension was then centrifuged at 400 x g for 4 minutes. The pellet of cells was resuspended in ice-cold harvesting solution (150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 50 mM boric acid, pH 7.2), centrifuged at 400 x g for 4 minutes, and then washed once more with harvesting solution in the same manner. The pellet of cells (6-8 ml total volume) was then suspended in an equal volume of harvesting solution (0° C) in preparation for the borate extraction procedure; equipment for the extraction was set up beforehand, so that cells did not remain in the harvesting solution for extended periods.

For the extraction of membranes, 300 ml of extraction solution (20 mM sodium borate, 0.2 mM EDTA, pH 9.6) at room temperature was equally divided between two 250 ml fluted Erlenmeyer flasks. The fluted flasks contained four inner fins of glass (see Figure 4) to create turbulence in the solution while stirring, which was important in order to obtain good membrane preparations. The solutions were stirred with a magnetic stirrer at a speed of 300 rpm. Then, while stirring, half of the previously mentioned cell suspension was slowly added down the side of each flask into the solution. Addition of the cells took about 2 minutes per flask. The flasks were stirred for another 5 minutes. During this time, plasma membrane ghosts peel away from the cytoplasmic constituents of the cell. The cytoplasm gels,

Figure 4. Fluted Erlenmeyer flask used in membrane preparations.



and aggregation between the "demembranized" cells occurs, forming jelly-like masses in the flask. After stirring, 6 ml of a 500 mM solution of sodium borate, pH 9.6, was added to each flask, and the jelly-like masses were removed by filtration through a nylon sieve. The solution was centrifuged at 900 x g for 5 minutes to remove small aggregates of membrane-free cells, connective tissue pieces, and other debris. The supernatant was then centrifuged at 9,000 x g, at 4° C, for one hour. The membrane ghosts sediment in the pellet, and the supernatant was discarded. To remove contaminating organelles, notably mitochondria, the pellet was resuspended in 5-10 ml of an ice-cold solution containing 20 mM sodium borate, 1 mM EDTA, pH 9.2. This suspension was carefully layered on top of a 35% (w/w) aqueous sucrose solution, and centrifuged at 25,000 x g for one hour at 4° C on a Beckman L2-65B ultracentrifuge. Plasma membranes remained at the top of the sucrose solution, which acts as a cushion; mitochondria and other organelles were found in the bottom pellet. The plasma membrane fraction was removed from the top of the sucrose cushion, and resuspended in 80 ml of ice-cold distilled water. This suspension was then centrifuged at 4° C at 10,000 x g for one hour. The resultant pellet, composed primarily of plasma membranes, was routinely resuspended in 0.5 to 1.0 ml of 0.1 M cacodylate-HCl buffer, pH 5.5. This yielded a concentration of membrane protein of 0.8-3.0 mg/ml, as tested by the procedure of Lowry et al. (1951). This solution was frozen at -20° C for 1-3 days, until sialyltransferase assays were performed. The pellet was occasionally resuspended in other buffers, depending upon the particular assay being performed. This procedure is schematized in Figure 5.

Figure 5. Flow chart for the preparation of plasma membranes.

LIVERS

↓

Dissociation by pipetting in CMF Tyrode's + 2 mM EDTA.

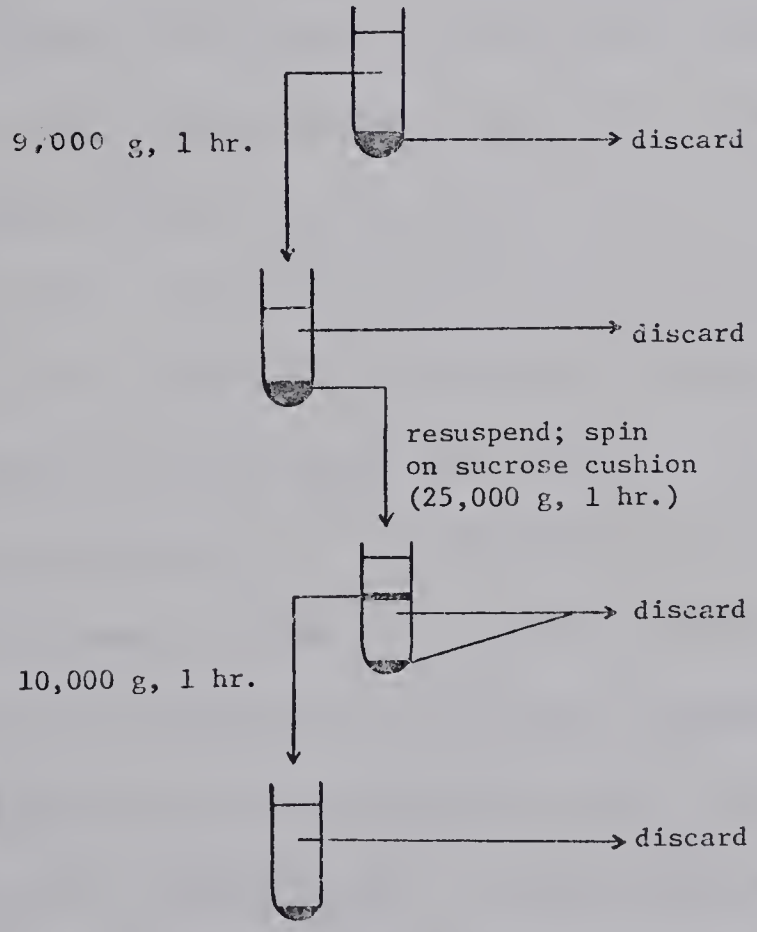
↓

Wash cells in Harvesting solution (150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 50 mM boric acid, pH 7.2.

↓

Extraction with stirring (20 mM NaBorate, 0.2 mM EDTA, pH 9.6).

↓ 900 g, 5 min.



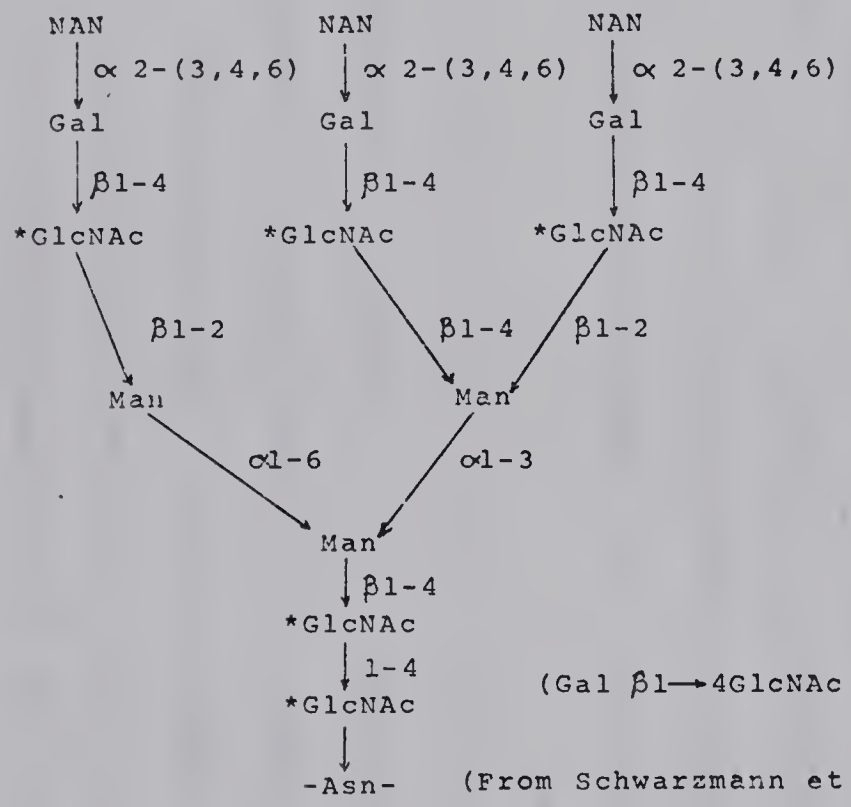
Substrate Preparation

Cytidine 5'-monophosphate sialic acid (sialic acid-4,5,6,7,8,9-^{14}C), of a specific activity of 276 mCi/mmole, and cytidine 5'-monophosphate sialic acid (sialic acid-4-^{14}C), of a specific activity of 1.67 mCi/mmole were purchased from New England Nuclear. Fetuin was obtained from the Grand Island Biological Company (GIBCO). Human α_1 acid glycoprotein was a generous gift of Dr. Y. L. Hao, National Fractionation Center, American National Red Cross, Bethesda, Maryland. Lactose, D-galactopyranosyl-β-thio-galactopyranoside (β-thiodigalactoside), and phenyl-β-D-galactopyranoside were obtained from Sigma Chemical Company. N-acetyllactosamine (βgal 1→4glcNAc) and the chemically synthesized "glycoproteins," were the generous gifts of Dr. R. U. Lemieux, Department of Chemistry, University of Alberta. The structures of the glycoprotein acceptors are shown in Figure 6. Other chemicals were obtained commercially, and were of analytical grade.

Free sialic acid was tested for by the procedure of Warren (1959), which was performed as follows. To tubes containing 2-20 μg of sialic acid in 0.2 ml water was added 0.1 ml of a solution containing 0.2 M sodium metaperiodate in 9 M phosphoric acid. The contents were mixed, and allowed to stand at room temperature for 20 minutes. Then, 1 ml of a solution containing 10% sodium arsenite, 0.5 M sodium sulfate and 0.1 N H_2SO_4 was added to each tube, and the tubes shaken until the yellow-brown color disappeared. To each tube was then added 3 ml of a solution containing 0.6% thiobarbituric acid and 0.5 M sodium sulfate. The contents were mixed, and the tubes were capped and heated in a boiling water bath for 15 minutes. After cooling in a water bath for

Figure 6. Acceptor structures. In α_1 acid glycoprotein, the * represents a glcNAc to which another galß1→4glcNAc may be attached. Fetuin contains two types of carbohydrate units, one linked to asparagine and another linked to serine or threonine.

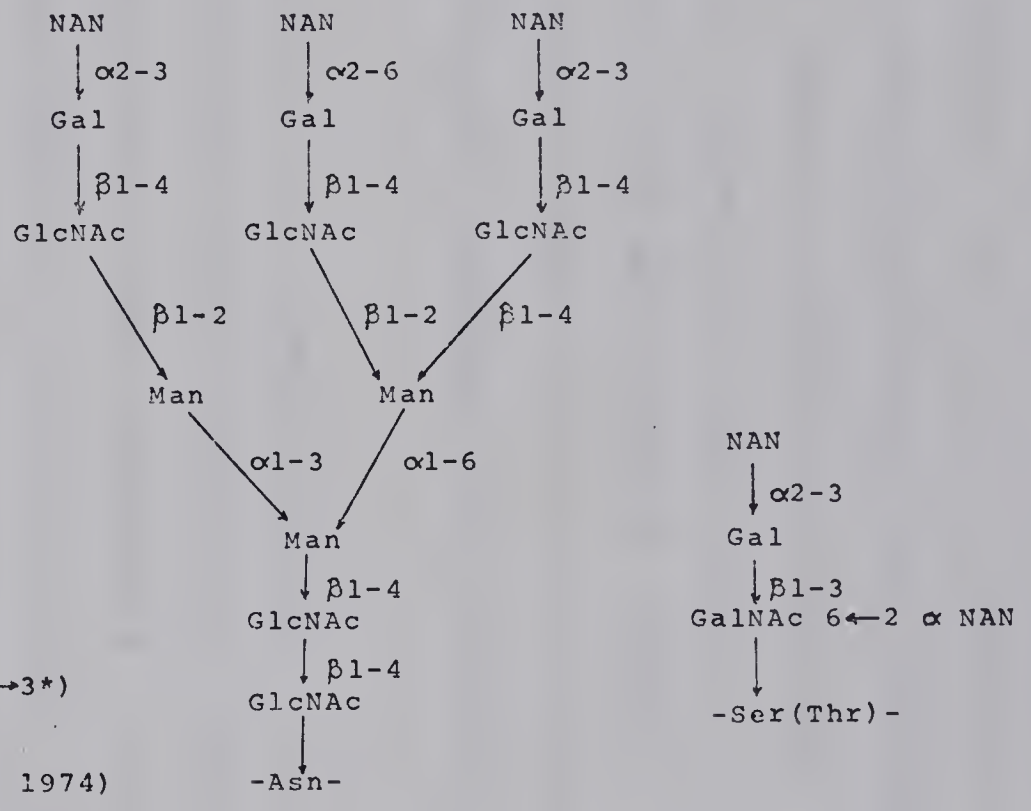
α_1 acid glycoprotein



(Gal β1→4GlcNAc β1→3*)

(From Schwarzmann et al, 1974)

Fetuin



(From Spiro and Bhoyroo, 1972 and Baenziger and Fiete, 1979)

Chemically synthesized "glycoproteins"

$$[gal\beta1\rightarrow4glcNAc\beta\text{-}O\text{-}(CH_2)_8\overset{O}{\overset{\|}{C}}N]_{16}\text{-}BSA$$

$$[gal\beta1\rightarrow3glcNAc\beta\text{-}O\text{-}(CH_2)_8\overset{O}{\overset{\|}{C}}N]_{26}\text{-}BSA$$

$$[gal\beta1\rightarrow3galNAc\alpha\text{-}O\text{-}(CH_2)_8\overset{O}{\overset{\|}{C}}N]_{14}\text{-}BSA$$

(Synthesized by the laboratory of Dr. R.U. Lemieux; BSA=Bovine Serum Albumin)

5 minutes, 4.3 ml of cyclohexanone was added to each tube. The pinkish-red color was extracted into the cyclohexanone by shaking the tubes vigorously. After extraction, the tubes were centrifuged for 5 minutes at 1,000 x g. The pink cyclohexanone phase was transferred to cuvettes, and the optical density determined at 549 nm. Standards containing 2-20 µg of sialic acid were run with each determination, and the amount of sialic acid present in samples was determined by comparison to the standard curve.

The Worthington Galactostat test (Worthington Biochemical Company, Freehold, New Jersey) was used to test for terminal galactosides present on glycoprotein substrates. This test is based on the ability of galactose oxidase (Avigad et al., 1962) to oxidize galactose at the 6 position, producing one mole of hydrogen peroxide per mole of galactose oxidized. A peroxidase then utilizes the hydrogen peroxide to oxidize a chromogen substrate (O-tolidine), which then absorbs at 425 nm. Samples are compared to standards containing known amounts of galactose.

Fetuin and α_1 acid glycoprotein were desialyzed according to the method of Spiro (1966). Glycoproteins were suspended in distilled water at a concentration of 6 mg/ml. An equal volume of 0.2 N H_2SO_4 was added, and the solution was heated to 80° C for one hour. The solutions were neutralized with NaOH and exhaustively dialyzed, first against 0.1 M NaCl (3 changes) and then against distilled water (at least 5 changes) to remove salts and free sialic acid. All dialyses in this entire report were carried out at 4° C, unless otherwise mentioned. Aliquots of the mixtures before and after dialysis were

tested for free sialic acid by the method of Warren (1959), and terminal galactose in desialyzed fetuin was tested for by the Worthington Galactostat assay. The desialyzed product was recovered by lyophilization, and stored desiccated at -20° C. Liberated sialic acid routinely ranged between 13 and 15 moles per mole of fetuin, and 13 and 14 moles per mole of α_1 acid glycoprotein. Galactoside termini ranged between 10 and 11 moles per mole of desialyzed fetuin; no terminal galactose could be detected in the native fetuin.

The above determinations were used as a semi-quantitative estimate only of the number of terminal galactoside residues on the glycoprotein. Not all galactosides in these glycoproteins necessarily have a sialic acid attached to them, due to microheterogeneity in their structures. Therefore, the amount of liberated sialic acid is usually less than the actual amount of exposed terminal galactosides after removal of the sialic acid. The Galactostat test is also inaccurate in quantitating galactoside termini, since Avigad et al. (1962) have shown that galactose oxidase, which is used in this test, oxidizes various galactosides at markedly different rates than it does galactose. Values used for galactoside termini per molecule of glycoprotein were the values reported by Spiro (1973) for fetuin (14) and Yamashina (1956) for human α_1 acid glycoprotein (16). In these studies the numbers of galactose residues present were chemically quantitated.

Desialyzed agalactofetuin was prepared from desialyzed fetuin by a single Smith Degradation (Smith and Van Cleve, 1955; Goldstein et al., 1959, 1965). Spiro (1964) has shown that the carbohydrate units of fetuin remain attached to the protein core after a single Smith

degradation. Removal of galactose by the procedure of Spiro (1966) is selective, leaving N-acetylglucosaminosides at the termini. The procedure is outlined as follows. Desialyzed fetuin (300 mg) was dissolved in 100 ml of an ice-cold solution containing 0.05 M sodium metaperiodate and 0.05 M sodium acetate, pH 4.5, in the dark. This solution was kept in the dark at 4° C for 10 hours, after which the pH was checked and found to be pH 4.6. Ethylene glycol (400 µl) was added to reduce excess periodate, and the solution was dialyzed exhaustively, first against 0.1 M NaCl, then against distilled water. The solution of about 100 ml was brought to 0° C, and was then mixed with 100 ml of a solution containing 0.3 M sodium borohydride and 0.3 M sodium borate, pH 8.0, also at 0° C. The mixture was kept on an ice-water bath for 12 hours; during this time the pH rose to 8.9-9.1. The pH was lowered to pH 5.0 with glacial acetic acid, and the solution was dialyzed exhaustively, first against 0.1 M NaCl, then distilled water. The solution was then concentrated to approximately 100 ml by placing the dialysis bag on polyethylene glycol 20,000 (4° C). To this solution 100 ml 0.1 N H_2SO_4 was added, and the mixture was heated to 80° C for one hour. The solution was neutralized with NaOH, and dialyzed against distilled water, then frozen and lyophilized. No galactose could be detected on this molecule by the Galactostat test.

Sialyltransferase Assay

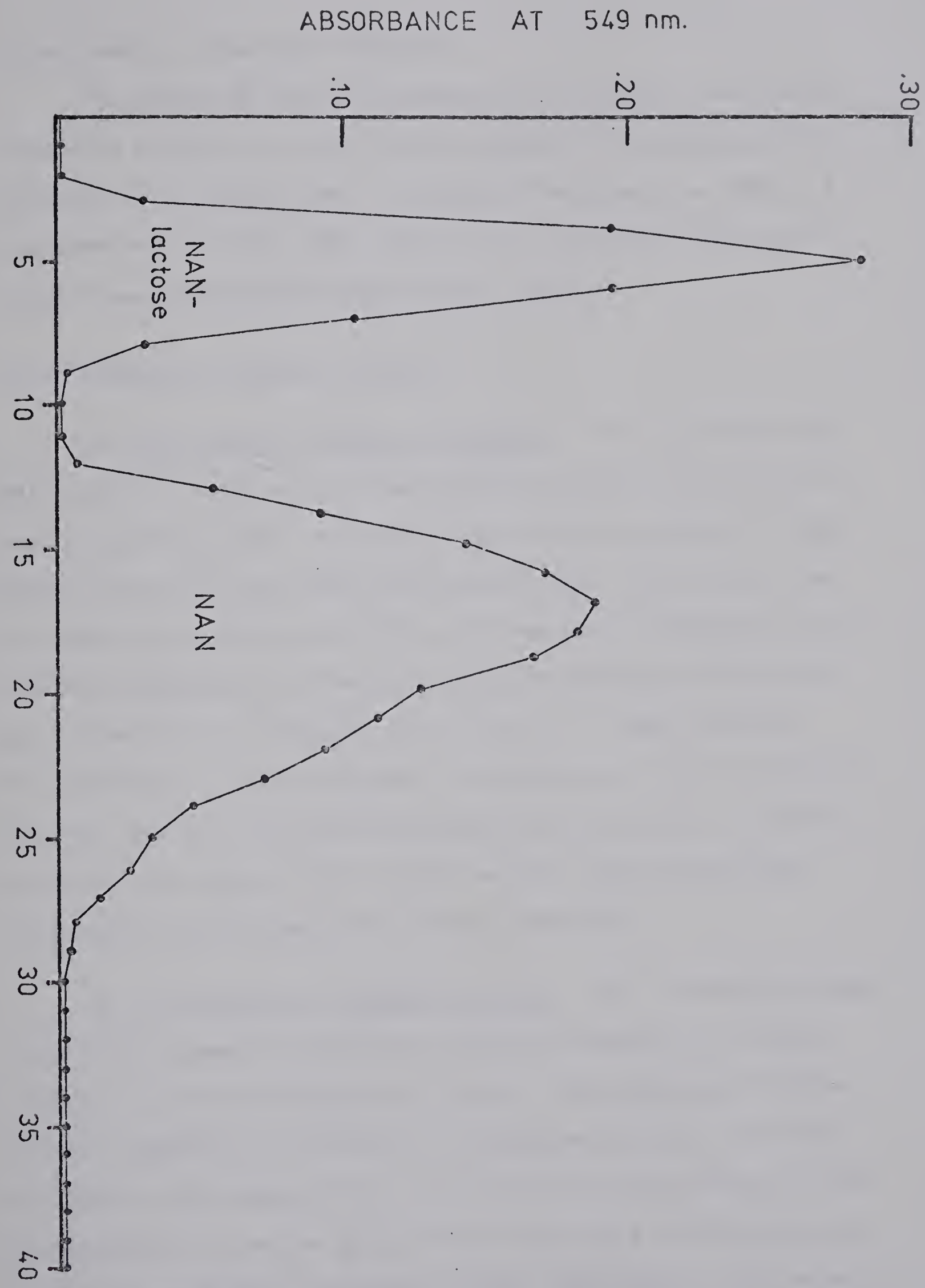
Unless otherwise mentioned, the assay procedure used for the quantitation of sialyltransferase activity was performed as follows. The reaction mixture consisted of a total volume of 100 µl, containing

40-150 µg of plasma membrane protein, 2 mM terminal galactoside acceptor, 0.6 mM ^{14}C-cytidine-5'-monophosphate-N-acetylneuraminic acid (^{14}C-CMP-NAN), 1.0% Triton X-100, and 0.05 M cacodylate-HCl, pH 5.5. The solution was briefly mixed on a vortex mixer, and incubated at 30° C for 20 minutes. Desialyzed α_1 glycoprotein was used as the routine acceptor in all assays except specificity studies, because this glycoprotein contains terminal galactosides which are only linked β1 →4 to glcNAc. After the 20 minute incubation, the solution was quickly cooled by placing the tubes into crushed ice for one minute. The amount of ^{14}C-NAN transferred to acceptors was determined by two different methods.

A. If the acceptor was a glycoprotein or chemically synthesized "glycoprotein," 0.5 ml of an ice-cold solution containing 15% trichloroacetic acid (w/v) and 5% phosphotungstic acid (w/v) were added to each tube (Paulson et al., 1977a). Tubes were centrifuged at 700 x g for 2 minutes (0° C) and the resultant pellet was resuspended and washed three more times with the same solution. Care was taken to maintain the temperature at 0° C at all times, in order to avoid the hydrolysis of the sialoside linkage, which occurs under acid conditions at higher temperatures. This linkage was found to be stable under the above conditions, since no release of NAN from NAN-lactose could be detected. The final pellet was dissolved in 0.5 ml of NCS solubilizer (Amersham) and counted in 10 ml of a 0.4% 2,5-diphenyloxazole (PPO), 0.0125% p-Bis(2-(5-phenyloxazolyl))-benzene (POPOP) solution in toluene (all reagents of scintillation grade). Resultant counts in all assays were subtracted from controls conducted without exogenous acceptors.

B. If the acceptor galactoside was of low molecular weight (lactose, N-acetyllactosamine, β-phenylgalactoside, and β-thio-digalactoside) the routine procedure used to determine the amount of sialic acid transferred was as follows. All procedures were carried out at 4° C. A series of columns (16.5 x 0.5 cm) of Dowex AG-2-X8 (Cl^- form, Bio-Rad Laboratories) were prepared. They were well washed, first with 3 M NaCl, then with 15 mM Tris-HCl, pH 7.0. The previously incubated sialyltransferase assay mixture was kept 1-3 minutes on ice and then placed on top the column, and run into the resin, collecting the eluant. The top was carefully rinsed with 15 mM Tris-HCl, pH 7.0, while the column was still running, and the column was washed with the same buffer. The first 5 ml eluted from the column was collected and counted in 15 ml Aquasol (New England Nuclear). This procedure separates the sialoside produced in the assay, which is eluted in the first 5 ml from both NAN, which elutes later than the sialoside, and CMP-NAN, which is strongly bound to the column. Figure 7 shows the separation of NAN from NAN-lactose using this procedure. NAN-lactose was quantitated by testing for sialic acid released after a mild acid hydrolysis (80° C, 0.1 N H_2SO_4, 1 hr). Both compounds were obtained from Sigma Chemical Company. Separation of NAN from the sialosides is essential in this assay, since a small amount of NAN is released from CMP-NAN under the conditions of the assay. It has been reported (Comb et al., 1966) that about 20% of a solution of CMP-NAN is hydrolyzed to NAN and CMP in one hour when treated at pH 5.5 and 37° C. Counts per minute obtained in controls run without exogenous acceptor galactosides were subtracted from experimental values. The assay procedures are

Figure 7. Separation of NAN and NAN-lactose on Dowex AG2-X8 columns (Cl^- form, 16.5 x 0.5 cm, washed with 15 mM Tris-HCl, pH 7.0, 4°C). Fractions (0.5 ml) were collected and tested for NAN by the procedure of Warren (1959), and the absorbance of the chromagen was plotted on the ordinate. NAN-lactose was determined by hydrolysis of samples in mild acid, and testing for liberated NAN by the same procedure.


ABSORBANCE AT 549 nm.
.10
.20
.30
NAN-lactose
NAN
5
10
15
20
25
30
35
40
FRACTION NO.

schematized in a flow chart (Figure 8).

The effects of nucleotide phosphates on the enzyme activity were studied by adding to the assay mixture cytidine-5'-triphosphate (CTP), cytidine-5'-diphosphate (CDP), or cytidine-5'monophosphate (CMP) to a concentration of 1.0 mM. CMP (disodium salt), CDP (type III) and CTP (type V) were obtained from Sigma Chemical Company.

Characterization of Labelled Product

A. Separation of ^{14}C-labelled product. ^{14}C incorporated into high molecular weight material was obtained by labelling desialyzed α_1 acid glycoprotein with ^{14}C-CMP-NAN of high specific activity (276 mCi/mmole); 0.05 µCi of the latter was used per assay. The mixtures were incubated for one hour as described, and were passed through 18 x 0.5 cm columns of Sephadex G-75 (Pharmacia Fine Chemicals), pre-equilibrated and eluted with 15 mM tris-HCl, pH 7.0, at 4° C. Fifty fractions (3 drops/fraction) were collected, 5 µl aliquots were removed from each fraction, and the ^{14}C present was counted in 10 ml Aquasol. Fractions containing the large molecular weight material, which eluted first, were pooled (total volume of 0.7 ml) and lyophilized.

B. Neuraminidase treatment of product. The ^{14}C-labelled product above was resuspended in 350 µl of a solution containing 0.1% $CaCl_2$, 0.9% NaCl, and 50 mM sodium acetate, pH 5.5. Ten microliters of this solution contained 3.2 picomoles of ^{14}C-labelled sialoside (1,940 dpm of ^{14}C-NAN of 612 dpm/picomole). To 10 µl of the above solution on ice was added 10 µl of an ice-cold solution containing 0.010 International Units of neuraminidase from *Vibrio cholerae* (Behringwerke, West Germany),

Figure 8. Procedure for the assay of sialyltransferase activity.

Incubate acceptor, membranes, and ^{14}C-CMP-NAN in 1% Triton X-100, 50 mM cacodylate-HCl, pH 5.5 for 20 min. at 30°C

Small M. W. acceptors

Quickly cool to 0°C, pass through Dowex 2-X8 column at 4°C

↓

Collect first 5 ml

↓

Count in Aquasol (gel state)

Large M. W. acceptors

Precipitate large M. W. material with 15% TCA, 5% PTA at 0°C

↓ 700 g, 2 min., 0°C

Wash pellet 3 more times with 15% TCA, 5% PTA, 0°C

↓

Dissolve pellet in 0.5 ml NCS

↓

Count in PPO-POPOP-toluene

also dissolved in the same buffer solution. After a brief mixing with a vortex mixer, tubes were incubated at 37° C for increasing time intervals up to 2 hours. Following incubation, the tubes were cooled for 1 min on ice, and to each tube was added 100 µl of an ice-cold solution containing 15% trichloroacetic acid, 5% phosphotungstic acid. Tubes were mixed with a vortex mixer, and centrifuged for 1 min at 5,000 x g at 4° C. The supernatant, containing free sialic acid, was removed and counted in 10 ml Aquasol. Controls conducted without enzyme were also performed, to find out whether the conditions under which the assay was run resulted in release of sialic acid.

C. Linkage characterization

1. ^{3}H labelling of galactoside termini of desialyzed α_1 acid glycoprotein

The procedure used to label galactoside termini selectively at the 6 position was essentially that outlined by Morell et al. (1966), as described by Van den Eijnden et al. (1977). Forty-nine milligrams of desialyzed α_1 acid glycoprotein was dissolved in 10 ml of a solution containing 50 mM NaCl in 50 mM sodium phosphate buffer, pH 7.0. To this solution 125 units of galactose oxidase were added (Sigma Chemical Company, type IV, where one unit is defined as the amount of enzyme which will produce a change in absorbance at 425 nm of 1.0 per minute at pH 6.0 at 25° C, in a peroxidase and o-tolidine system of a total volume of 3.4 ml). Toluene (100 µl) was added to prevent bacterial growth, and the mixture was incubated at 37° C for 24 hours. Following incubation, the mixture was cooled to room temperature, and 15 ml of a solution containing 50 mM NaCl in 50 mM sodium phosphate buffer (pH 7.8)

was added. All further labelling procedures were carried out in a fume hood, and all dialyses were carried out at 0° C. To the mixture was added 50 mCi of NaB^3H_4 of a specific activity of 6.7 Ci/mmole (Amersham) in 100 µl of 0.01 N NaOH. Reduction was allowed to proceed for 45 minutes at room temperature. Then, 300 µmoles of $NaBH_4$ in 100 µl of the above buffer (pH 7.8) was added to the mixture. The solution was left at room temperature for 30 minutes; it was then dialyzed exhaustively, first against 0.1 M sodium acetate buffer, pH 5.6, and then against distilled water. The solution was then lyophilized, and any remaining borate was removed as methyl borate by five additions and evaporations of methanol. The resultant material was dissolved in 5 ml distilled water, and stored at -20° C. This material, labelled at the 6 position of galactose with tritium, was used as a substrate for the sialyltransferase.

2. Smith degradation procedure

In order to determine the linkage position of the sialic acid to galactose, the ^3H-labelled α_1 acid glycoprotein was used as an acceptor for ^{14}C-labelled NAN. For this experiment, NAN labelled at the C-4 position (specific activity of 1.67 mCi/mmole) was used. The Smith degradation procedure is outlined in Figure 9. Expected products were ^3H-glycerol, if NAN was linked to the 2 or 6 position of galactose, ^3H-threitol, if linked to the 4 position, and ^3H-galactose, if linked to the 3 position. Five to ten volumes of the sialyltransferase reaction mixture were used for these experiments, and the length of the incubation time was increased to one hour. The procedure for determining the linkage to galactose is a slight modification of the

Figure 9. The expected intermediates and products of the Smith degradation procedure, performed on:

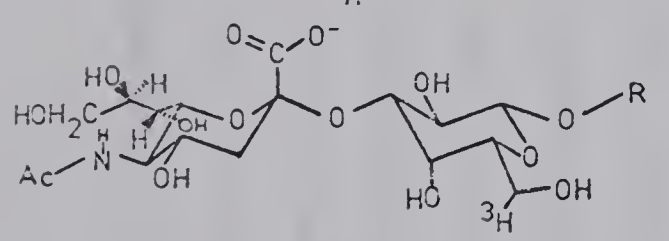
A. NANα2→3(6-^{3}H-gal)β-R.

B. (6-^{3}H-gal)β-R. If sialic acid is attached at either of the 2 or 6 positions of galactose, cleavage would still occur between carbons 3 and 4, giving rise to glycerol as well.

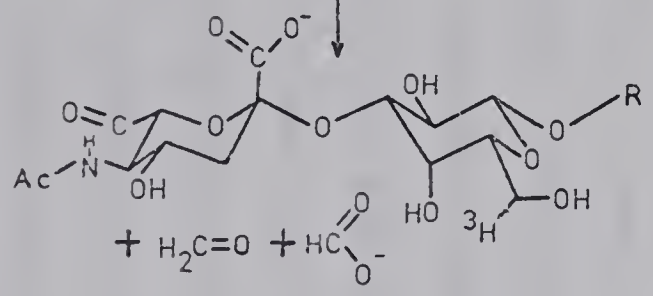
C. NANα2→4(6-^{3}H-gal)β-R. Cleavage would occur between carbons 2 and 3, giving rise to threitol.

Periodate oxidation results in oxidative cleavage between vicinal hydroxyl groups. The aglycone group, R, must be large enough to be retained during dialysis, as in the case of glycoproteins.

A



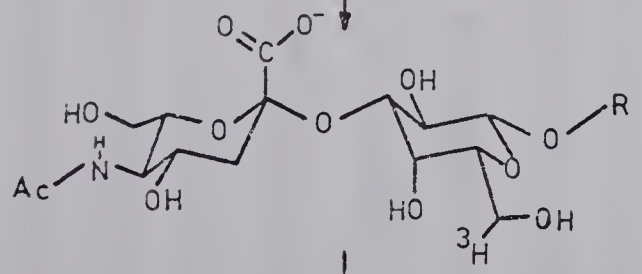
$NaIO_4$



+ $H_2C{=}O$ + $HC(=O)O^-$

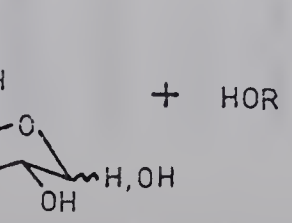
(1) Dialyze

(2) $NaBH_4$



(1) Dialyze

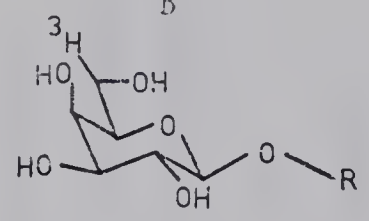
(2) H^+



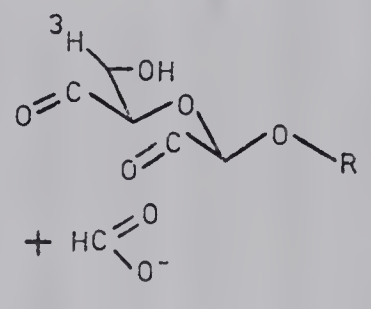
+ HOR

+ Degradation products of NAN

B



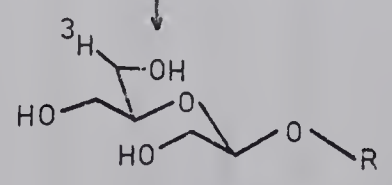
$NaIO_4$



+ $HC(=O)O^-$

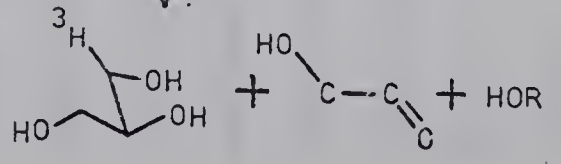
(1) Dialyze

(2) $NaBH_4$



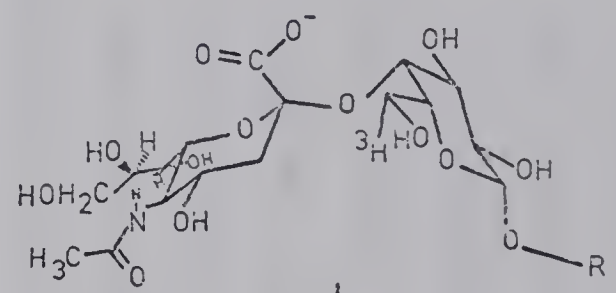
(1) Dialyze

(2) H^+

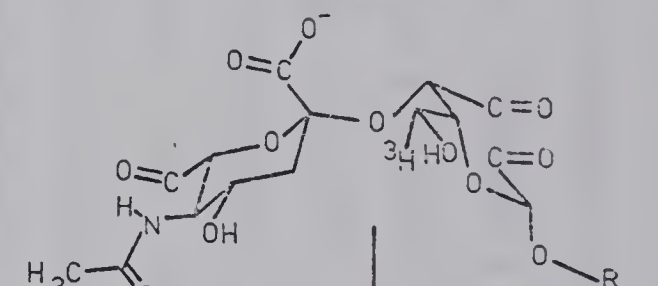


+ HOR

C

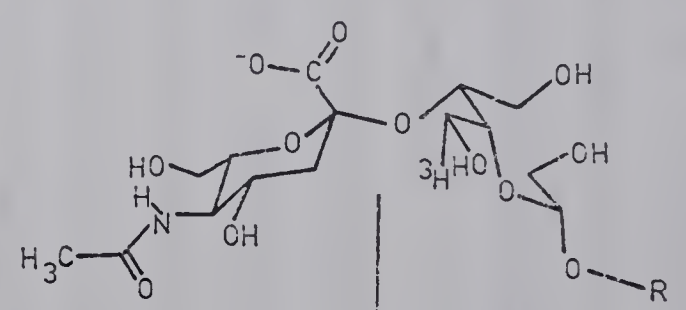


$NaIO_4$



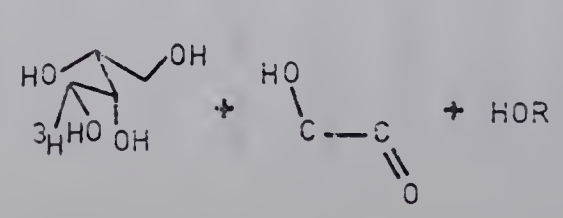
(1) Dialyze

(2) $NaBH_4$



(1) Dialyze

(2) H^+



+ HOR

+ Degradation products of NAN

Smith degradation as described by Spiro (1966).

After the sialyltransferase reaction, the mixture was cooled on ice. The entire procedure from this point on was performed at 4° C, unless otherwise mentioned. The sample was placed on a column of Sephadex G-75 (23.5 x 0.7 cm) which was pre-equilibrated with 0.05 M sodium acetate buffer, pH 4.5. The column was eluted with the same buffer; ten-drop fractions were collected. Aliquots (10 µl) from each fraction were dissolved in 10 ml Aquasol and counted simultaneously for ^{3}H and ^{14}C. Fractions containing the double-labelled large molecular weight material, which eluted first, were pooled. The pH was tested to make sure that it was at 4.5, because subsequent periodate oxidation was to be performed at this pH. A 10 µl aliquot of the pooled sample was counted in Aquasol (10 ml) for ^{3}H and ^{14}C, and the ratio of $^{3}H/^{14}C$ was determined. The volume of the solution was adjusted from approximately 1.8 ml to 2.0 ml with 0.05 M sodium acetate buffer, pH 4.5. Sodium metaperiodate was then added to a concentration of 0.1 M. The pH was checked at 4.5. The solution was well mixed, care being taken to keep the mixture in the dark, to prevent side reactions which occur in the presence of light. The tube was kept in the dark at 4° C for 10 hours. Then, 50 µl ethylene glycol was added in order to consume the remaining periodate, and after 30 minutes, the solution was exhaustively dialyzed, first against 0.1 M NaCl (3 changes), then against distilled water (at least 5 changes). After dialysis, the contents of the bag were removed and adjusted to a volume of 2.5 ml with distilled water. To the sample at 0° C was added 1 ml of a solution containing 0.4 M sodium borate and 0.4 M sodium borohydride,

pH 8.0. Reduction was allowed to proceed for 12 hours at 0° C. After this period the pH was lowered to 5.0 by adding glacial acetic acid. The sample was then dialyzed exhaustively, first against 0.1 M NaCl, then against distilled water. A 50 µl aliquot of this sample was counted for ^{14}C and ^{3}H in 10 ml Aquasol, and the $^{3}H/^{14}C$ ratio was determined, to compare it to the ratio obtained before the periodate oxidation/borohydride reduction procedure. To the dialyzed sample (approximately 4 ml) was added 1 ml of 10 N H_2SO_4, and hydrolysis was allowed to proceed in a sealed tube on a boiling water bath for 5 hours. To remove amino acids and peptides, the sample was then cooled to 4° C, and passed through a column of crushed Amberlite IR-120 (H^+ form, washed with water, 16 x 0.5 cm). The sample was eluted through the column with distilled water. The first 10 ml eluted from the column was passed through a Dowex AG2-X8 column (Bio-Rad, acetate form, 28 x 0.9 cm) which had previously been equilibrated with water. This was performed to replace sulfuric acid with acetic acid, which could be volatilized in the next step. The column was eluted with distilled water, and fifteen 5 ml fractions were collected. Aliquots (50 µl) were counted for ^{14}C and ^{3}H. Fractions containing ^{3}H were pooled, and the volume of liquid was brought to approximately 200 µl in a flash evaporator. This material was subjected to descending paper chromatography. The sample was applied at the origin on water-washed Whatman #1 chromatography paper, and the chromatogram was run in a descending fashion at 22° C with an n-butanol:ethanol:water (20:9:8) solvent mixture in a closed chromatography tank. Chromatograms were air dried and cut into 1 cm sections. Radioactivity in each section

was eluted by shaking the paper strips in 5 ml of water. The 5 ml sample was then counted in 15 ml of Aquasol. Controls, in which either no ^{14}C-CMP-NAN was added to the assay mixture, or in which no plasma membranes and no ^{14}C-CMP-NAN were added to the assay mixture, were performed as comparisons.

Standards of glycerol, threitol, and galactose, obtained commercially, were run on separate chromatograms. They were stained by spraying the chromatograms with 1% $AgNO_3$ in a 1:1 solution of NH_4OH:H_2O, and heating in an oven to 120° C (Partridge, 1948). A flow chart (Figure 10) schematizes the Smith degradation procedure as applied to the sialyltransferase product.

Assays for Marker Enzymes

Two enzymatic activities were previously noted by Sanford and Rosenberg (1972) to be enriched in plasma membrane fractions of 15-day chick embryo livers. These were a Mg^{++}-dependent ATPase, and a Mg^{++}-dependent CTPase, which were purified 15.6 and 13.5-fold, respectively, in plasma membrane fractions. In order to determine the degree of purification of plasma membrane material, the activities of these two enzymes were determined on homogenates of embryonic liver and on plasma membrane preparations obtained by the procedure described earlier.

CMP, type V, and Adenosine-5'-triphosphate (ATP), Sigma grade, were obtained from Sigma Chemical Company. The assay mixture consisted of a total volume of 1.5 ml, containing 3-6 µg membrane protein or 100-130 µg homogenate protein; either CTP or ATP, 3 mM; $MgSO_4$, 3 mM;

Figure 10. Flow chart of the Smith degradation procedure applied to the double-labelled product of the sialyltransferase reaction.

Double-labelled product

↓

Periodate Oxidation

↓

Borohydride Reduction

↓

Acid Hydrolysis

↓

Mixture passed through crushed Amberlite IR-120

↓

Mixture passed through Dowex 2

↓

Volume reduced to approximately 200 µl in a flash evaporator

↓

Chromatographed on washed Whatman # 1 paper (descending)

↓

Chromatograms dried and cut into 1 cm sections

↓

Radioactivity eluted from sections and counted in Aquasol

NaCl, 120 mM; KCl, 5 mM; and N-2-Hydroxyethyl-piperazine-N'-2-ethanesulfonic acid (HEPES), 10 mM, pH 7.2 (modification of Jorgensen, 1974). The mixture was incubated at 37° C for 30 minutes. Tubes were cooled on ice for 1 minute, and 1.5 ml of an ice-cold solution containing 10% (w/v) TCA was added to each sample. The tubes were centrifuged at 900 x g for 4 minutes to remove the protein precipitate, and free phosphate was tested for in the supernatant by the procedure of Fiske and Subbarow (1925). Controls consisted of assays conducted without nucleotide-5'-triphosphate, without enzyme, and without both of these components, and the amount of free phosphate obtained in controls (generally low) was subtracted from the amount present in the experimental samples. Protein was determined by the procedure of Lowry et al. (1951) or by the procedure of Bradford (1976). In some assays testing for ATPase activity, ouabain (Strophanthin-G, Sigma Chemical Company) was added to the incubation mixture at a concentration of 1.3 mM. Assays were run so that no more than 30% of the nucleotide-5'-triphosphate was utilized during the 30 minutes of the reaction.

Electron Microscopy

Preparation of sections for electron microscopy was kindly performed by Dr. E. J. Sanders, Department of Physiology, University of Alberta. Pellets of the plasma membrane preparation were fixed for one hour in a solution containing 3.5% glutaraldehyde in 0.1 M cacodylate-HCl buffer, pH 7.0. The pellets were then well washed with 0.1 M cacodylate-HCl buffer, pH 7.0. They were fixed with 1% osmium

tetraoxide in the same buffer (2 hr), and dehydrated in increasing concentrations of ethanol (50% to absolute, 10% intervals) leaving the sample 10 minutes at each concentration, and changing the final absolute ethanol solution twice to ensure complete dehydration. Samples were placed in propylene oxide (2 changes, 15 minutes in each), and left overnight in a 1:1 mixture of propylene oxide and Araldite. They were then embedded in Araldite, and placed in an oven at 64° C for 48 hours. Sections (60 nm in thickness) were cut, and stained with 5% uranyl acetate in absolute methanol for 15 minutes. They were then washed with 4 changes of absolute methanol, counterstained with lead citrate for 2 minutes, and well washed with distilled water. Sections were viewed with a Phillips 300 electron microscope.

RESULTS

Plasma Membrane Preparation

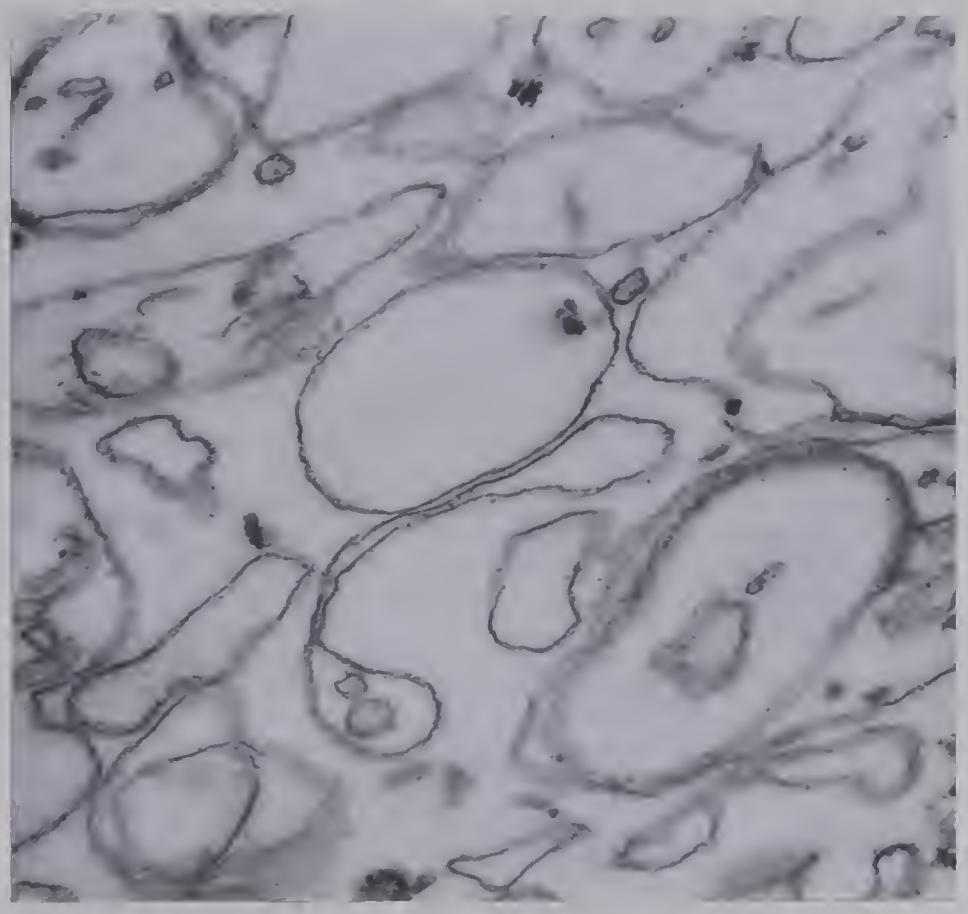
The livers of 13-day chicken embryos consist primarily of parenchymal cells, arranged radially around numerous bile ducts. Small vascular spaces are evident throughout the liver, and are surrounded by perivascular endothelial cells which are differentiating to form granulocytes (Romanoff, 1960). Most of the granulocytes, and other constituents of the blood present in livers, are probably removed by perfusing the livers, but endothelial cells likely remain. Hence, the plasma membrane preparations used in these experiments would be composed primarily, but not entirely, of plasma membranes of parenchymal cells.

An electron micrograph of the plasma membrane preparation is shown in Figure 11. The preparation consists mainly of membrane ghosts; no lysosomes or rough endoplasmic reticulum could be observed, and swollen mitochondria were scarce, but present.

Specific activities for the plasma membrane located enzymes (Mg^{++} dependent ATPase and Mg^{++} dependent CTPase) in liver homogenates and in the purified plasma membrane preparation are shown in Table 1. The Mg^{++} CTPase and Mg^{++} ATPase are purified 8.5-fold, and 6.8-fold, respectively, in the plasma membrane preparation. An 8.6-fold purification of the sialyltransferase in the plasma membrane preparation was achieved, as compared to homogenates. Addition of ouabain (1.3 mM) resulted in no inhibition of ATPase activity,

Figure 11, A and B. Electron micrographs of the plasma membrane preparation, magnified 44,000 times.

A



B

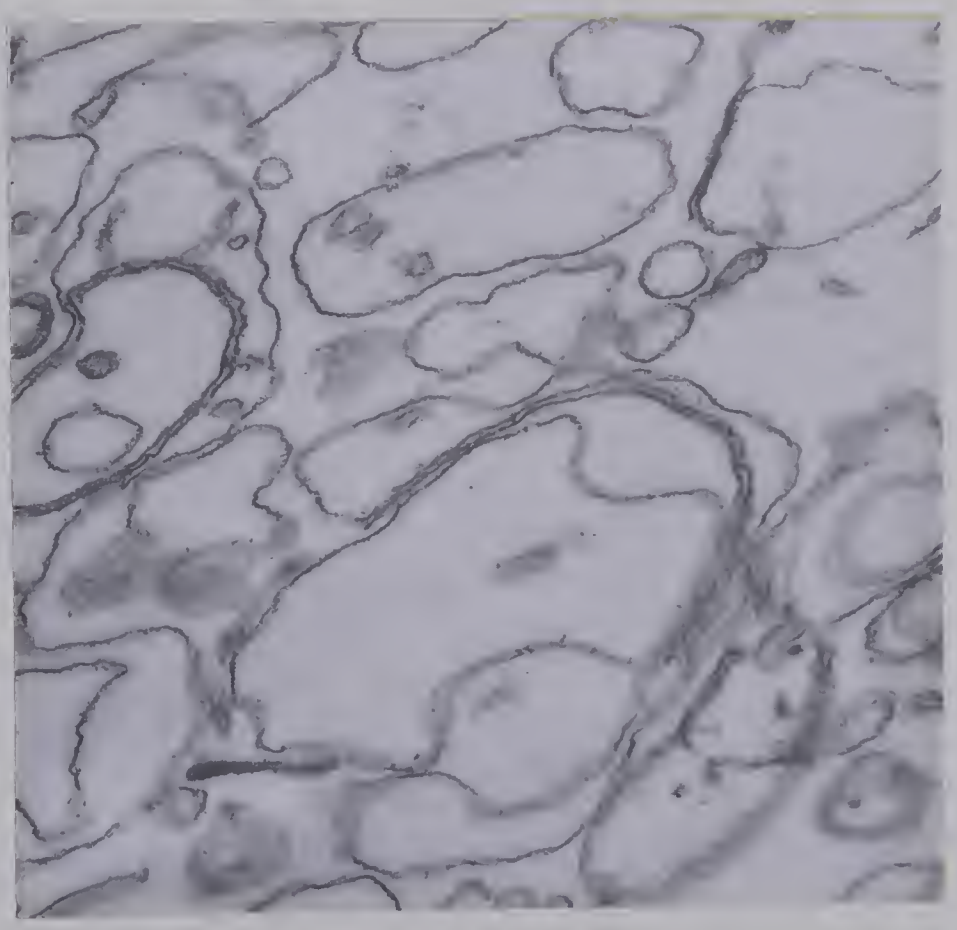


TABLE 1

ENZYME PURIFICATION IN PLASMA MEMBRANES

	ATPase		CTPase		Sialyltransferase	
	PLASMA MEMBRANES	HOMOGENATE	PLASMA MEMBRANES	HOMOGENATE	PLASMA MEMBRANES	HOMOGENATE
SPECIFIC ACTIVITY (UNITS*)	1.55 1.45	.222 .221	2.37 2.67	.292 .298	22.0 18.6 14.7 19.3	2.33 2.03
AVERAGE SPECIFIC ACTIVITY	1.50	.222	2.52	.295	18.7	2.18
PURIFICATION	6.76		8.54		8.58	

*1 Unit for the ATPase and CTPase is 1 μmole PO_4 released/min·mg prot.
1 Unit for the sialyltransferase is 1 nmole NAN transferred/hr·mg prot.

indicating that it is not the typical enzyme involved in the active transport of ions. This was also observed by Sanford and Rosenberg (1972) in their investigation of enzymes in embryonic liver plasma membranes. Succinic dehydrogenase, a mitochondrial marker enzyme, has not been detected in this plasma membrane preparation, but is present in the mitochondrial pellet, obtained after the centrifugation step on the sucrose cushion (Zalik, unpublished results).

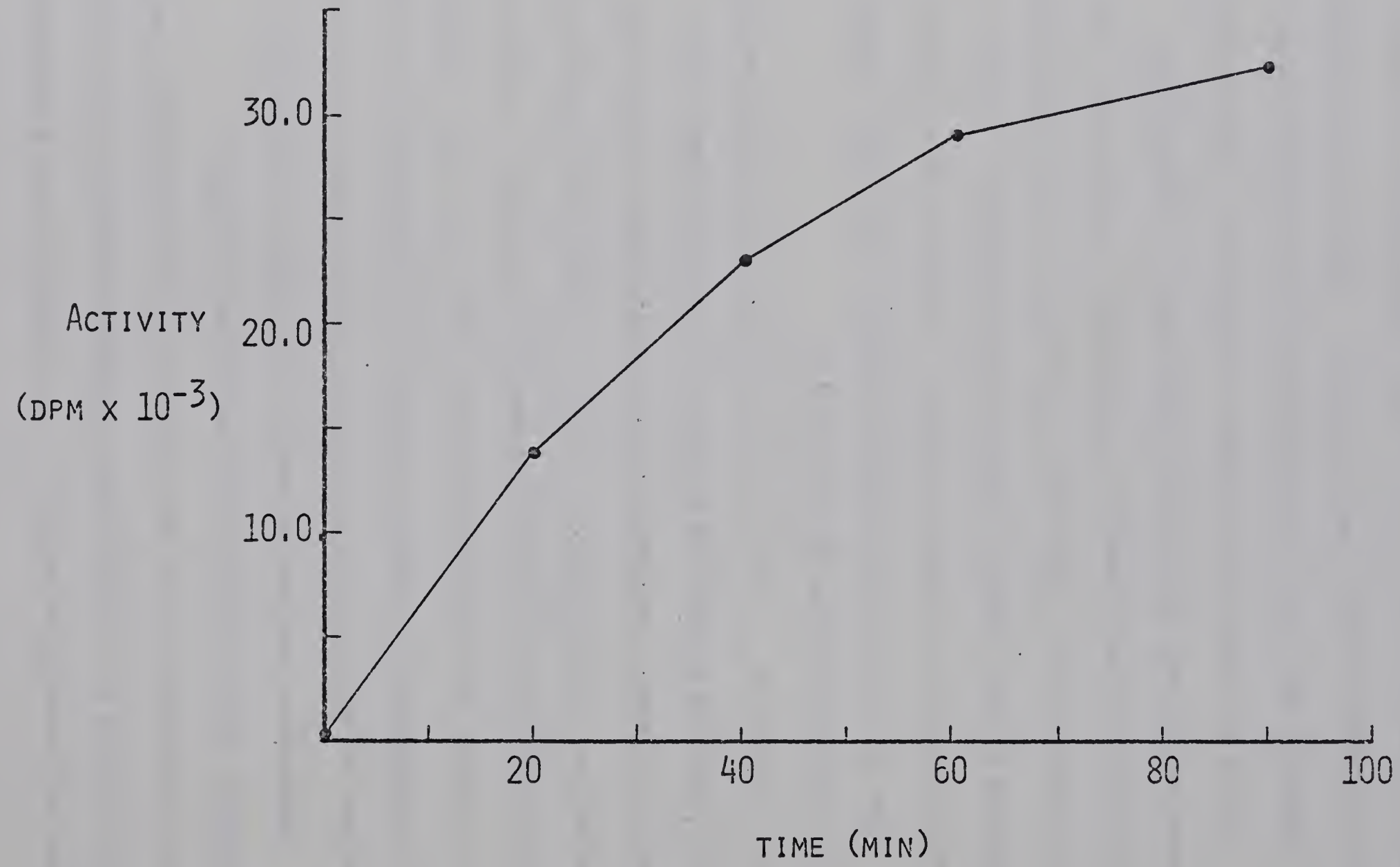
Sialyltransferase

Results reported for each study were obtained from duplicate experiments in which values never varied by more than ±7%. Experiments in which enzyme specificity was investigated were performed in triplicate and occasionally quadruplicate.

The time dependency of enzyme activity was determined at pH 7.0, using ^{14}C-CMP-NAN of a specific activity of 276 mCi/mmole (1.8 μM). As shown in Figure 12, the reaction occurs at nearly a constant rate for the first 20 minutes, and steadily decreases to about one-fifth the initial rate after one hour. All assays were therefore conducted for 20 minutes, when, judging from the slope of the curve, the activity was nearly linear with time. Less than 30% of the CMP-NAN was utilized during the 90-minute period of the reaction, indicating that the quantity of substrate was not a limiting factor in the reaction.

The detergent, Triton X-100, at a concentration of 1% (v/v), increases the activity of this membrane-bound transferase by about tenfold (Table 2). Most of the enzymatic activity is destroyed by boiling for one minute. Table 2 also shows that under the assay conditions, activity with endogenous acceptor was 23% of the activity

Figure 12. Time-dependent transfer of NAN to acceptor. Incubation mixtures consisted of membranes, galactoside termini on desialyzed α_1 acid glycoprotein (2 mM), ^{14}C-CMP-NAN (1.8 μM, 276 mCi/mmole), Triton X-100 (1%), and HEPES buffer (50 mM), pH 7.0. Each data point represents the average of duplicate assays, in which variation was never more than ±2.1% of the mean value.


30.0
20.0
10.0
ACTIVITY
(DPM X 10^{-3})
20
40
60
80
100
TIME (MIN)

obtained with saturating amounts of desialyzed α_1 acid glycoprotein. Controls testing for activity with endogenous acceptor were conducted with each individual assay, and this activity was subtracted from all experimental values obtained using exogenous acceptors.

Experiments designed to measure the effect of pH as well as temperature on enzyme activity were performed as described previously, using ^{14}C-CMP-NAN of a specific activity of 276 mCi/mmole (1.8 μM). Figure 13 shows the variation of activity with pH at 30° C (Figure 13A), and the variation of activity with temperature at pH 5.5 (Figure 13B). The enzyme has a pH optimum of 5.5, and a temperature optimum of 30° C. In studies in which the effect of pH was determined, HEPES buffer (0.05 M) was used between pH 7.0 and 8.0, cacodylate-HCl buffer (0.05 M) between pH 5.5 and 6.5, and sodium acetate-acetic acid buffer (0.05 M) was used between pH 4.0 and 5.0.

The effects of three divalent cations, Ca^{++}, Mg^{++}, and Mn^{++} on enzymatic activity are shown in Table 3. Assays were performed using ^{14}C-CMP-NAN of high specific activity (276 mCi/mmole, 1.8 μM). A 10 mM concentration of $MgCl_2$ had little effect on the enzyme activity, but activities in the presence of 10 mM $MnCl_2$ or 10 mM $CaCl_2$ were decreased to 83% and 53% of the control values, respectively.

Since for specificity studies, conditions of enzyme saturation were required, the effects of varying substrate concentrations on sialyltransferase activity were examined. When the concentration of CMP-NAN was varied, desialyzed α_1 acid glycoprotein was used at a constant "galactoside terminus" concentration of 1.0 mM. When the concentration of desialyzed α_1 acid glycoprotein was varied, ^{14}C-CMP-NAN

Table 2. Characteristics of the sialyltransferase assay. The complete system contained 40-150 μg membrane protein, 2 mM galactoside termini on desialyzed α_1 acid glycoprotein, 0.6 mM ^{14}C-CMP-NAN (1.67 mCi/mmole), 1% Triton X-100, and cacodylate-HCl buffer (50 mM), pH 5.5. The mixtures were incubated at 30°C for 20 minutes. Duplicate assays were averaged for each experiment, in which variation was never more than ±6.8% of the mean value.

Table 3. The effects of divalent cations on the reaction rate. The complete system was the same as described for Table 2, except 1.8 μM ^{14}C-CMP-NAN (276 mCi/mmole) was used. Duplicate assays were averaged for each experiment, in which variation was never more than ±6% of the mean value.

TABLE 2: ASSAY CHARACTERISTICS

SYSTEM	% MAXIMAL ACTIVITY
Complete system	100
Minus acceptor	23
Minus enzyme	3
Minus enzyme, plus heat-treated enzyme (boiled 1 min)	8
Minus Triton X-100	11

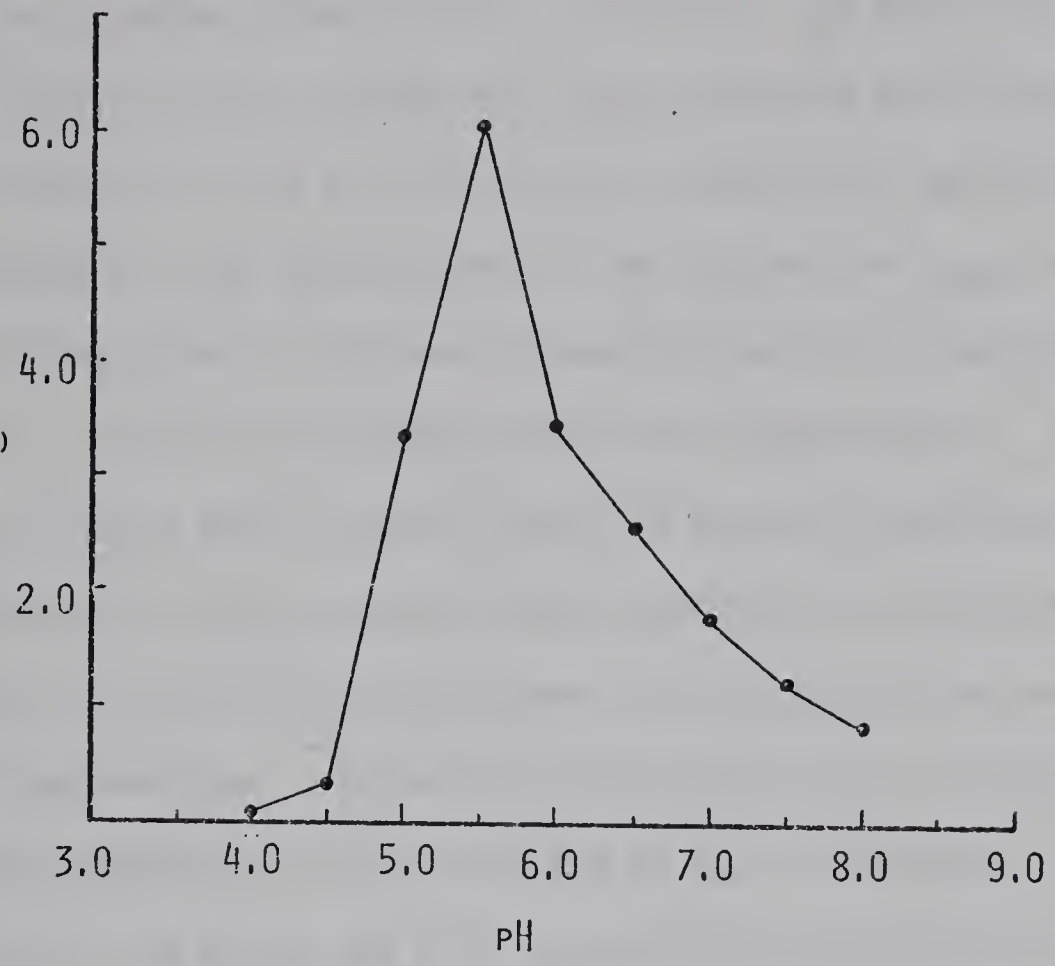
TABLE 3: EFFECTS OF DIVALENT CATIONS ON THE REACTION RATE

SYSTEM	% MAXIMAL ACTIVITY
Complete system	100
plus 10 mM $MgCl_2$	97
plus 10 mM $MnCl_2$	83
plus 10 mM $CaCl_2$	53

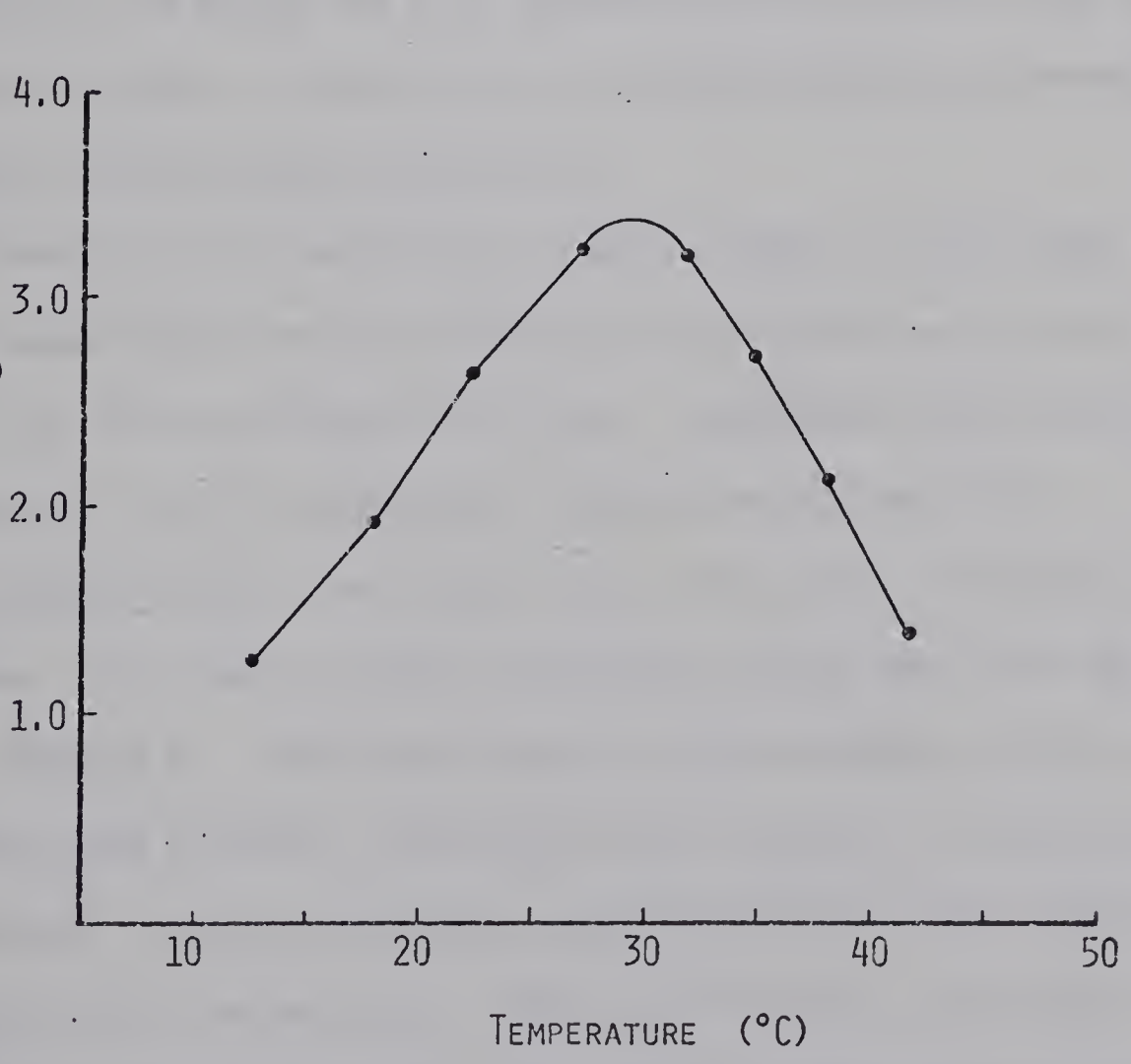
Figure 13A. Variation of sialyltransferase activity with pH. Incubation mixtures contained 40-150 µg membrane protein, 2 mM galactoside termini on desialyzed α_1 acid glycoprotein, 1.8 µM ^{14}C-CMP-NAN (276 mCi/mmole), 1% Triton X-100, and either HEPES, cacodylate-HCl, or sodium acetate-acetic acid buffer (50 mM) at various pH's. The mixtures were incubated at 30°C for 20 minutes. Each data point represents the average of duplicate assays, in which variation was never more than ±2.8% of the mean value.

Figure 13B. Variation of sialyltransferase activity with temperature. Incubation mixtures contained 40-150 µg membrane protein, 2 mM galactoside termini on desialyzed α_1 acid glycoprotein, 1.8 µM ^{14}C-CMP-NAN (276 mCi/mmole), 1% Triton X-100, and 50 mM cacodylate-HCl buffer, pH 5.5. The mixtures were incubated at different temperatures for 20 minutes. Each data point represents the average of duplicate assays, in which variation was never more than ±3.2% of the mean value.

13A


6.0
4.0
2.0
Activity
(dpm x 10^{-3})
3.0
4.0
5.0
6.0
7.0
8.0
9.0
pH

13B


4.0
3.0
2.0
1.0
Activity
(dpm x 10^{-3})
10
20
30
40
50
TEMPERATURE (°C)

was used at constant concentration of 0.6 mM. As shown in Figures 14 and 15, under the assay conditions, the enzyme is half-saturated at concentrations of 17 μM for CMP-NAN, and 180 μM for galactoside termini on desialyzed α_1 acid glycoprotein. The values for V_{max}, obtained from the reciprocal plots in Figures 14 and 15, were 19.2 nmoles/mg protein·hr, and 22.3 nmoles/mg protein·hr, respectively. As the data for these figures were obtained from two separate membrane preparations, this difference in V_{max} obtained per milligram of protein may reflect variations in the relative abundance of this particular enzyme within membrane preparations. Saturating conditions that were used for subsequent specificity studies were 0.60 mM for CMP-NAN, which corresponds to 36 times the half-saturating value, and 2.0 mM for galactoside termini on desialyzed α_1 acid glycoprotein, corresponding to 11 times the half-saturating value.

Results of the specificity studies (Table 4) show that, at a terminal galactoside concentration of 2 mM, desialyzed α_1 acid glycoprotein was the best acceptor for NAN. Desialyzed fetuin was the second best acceptor, and the chemically synthesized glycoprotein galβ1→4glcNAcβ-R-BSA (see Figure 6 for the complete structure), the third best. All three of these molecules contain βgal 1→4 βglcNAc termini (Figure 6). The disaccharide, galβ1→4glcNAc, is also a relatively good acceptor, and galβ1→4glc (lactose) is utilized to a lesser extent. Another molecule, galβ1→3 galNAcα-R-BSA (Figure 6) could also act as an acceptor. Other galactosides, including β-phenyl-galactoside, thiodigalactoside, and galβ1→3glcNAcβ-R-BSA (Figure 6), were not acceptors. Fetuin and desialyzed, agalactofetuin, present at

Figure 14. Variation of the reaction velocity with the concentration of CMP-NAN. S represents the concentration of CMP-NAN, and V represents the rate of transfer of NAN to desialyzed α_1 acid glycoprotein. Each data point represents the average of duplicate assays, in which variation was never more than ±6.5% of the mean value.

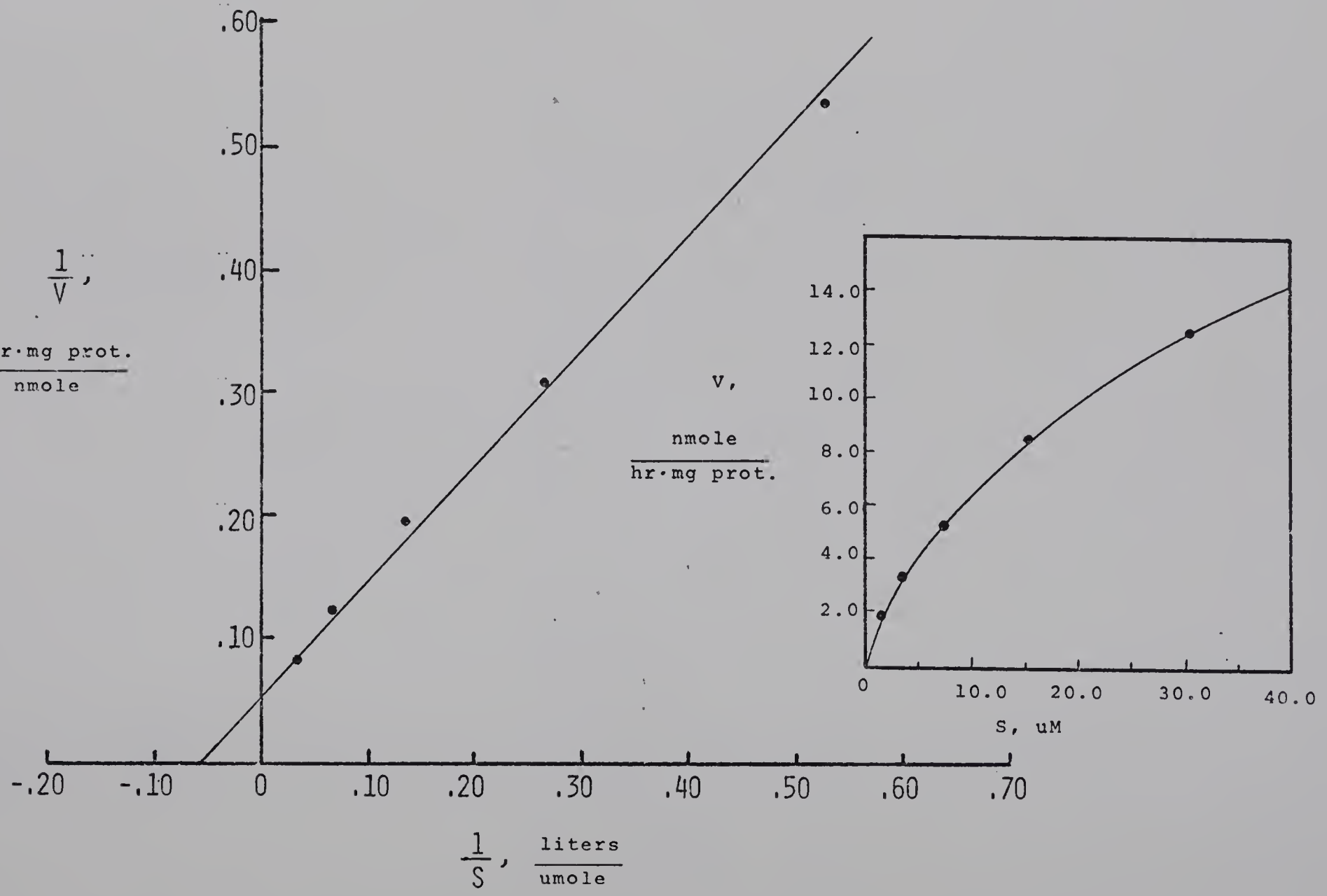

1/V, hr·mg prot./nmole
.60
.50
.40
.30
.20
.10
-.20
-.10
0
.10
.20
.30
.40
.50
.60
.70
1/S, liters/umole
V, nmole/hr·mg prot.
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0
10.0
20.0
30.0
40.0
S, uM

Figure 15. Variation of the reaction velocity with the acceptor concentration. S represents the concentration of galactoside termini on desialyzed α_1 acid glycoprotein in the reaction mixture. V represents the rate of transfer of NAN to the acceptor. Each data point represents the average of duplicate assays, in which variation was never more than ±5.7% of the mean value.

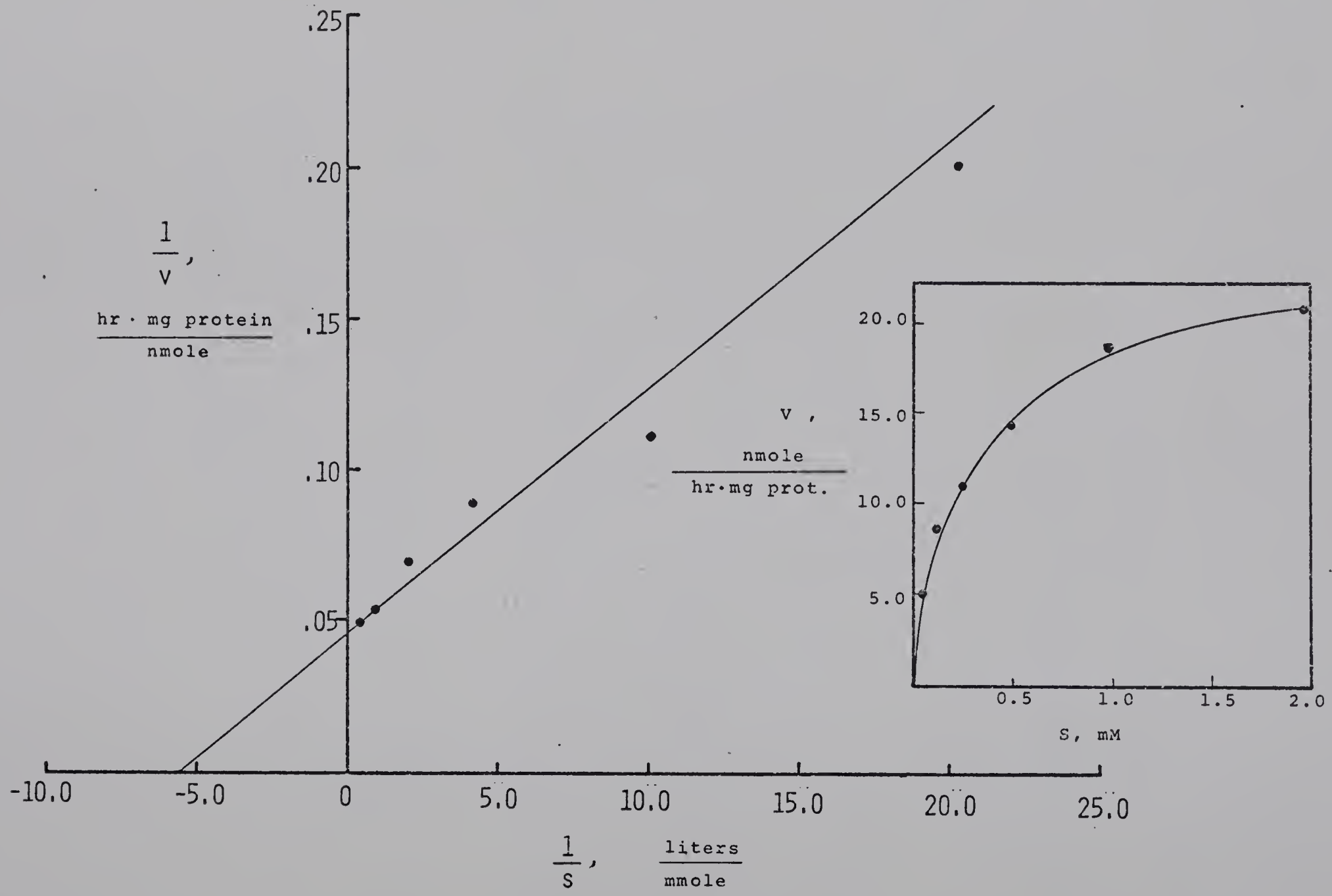

.25
.20
.15
.10
.05
1/V, hr · mg protein / nmole
-10.0
-5.0
0
5.0
10.0
15.0
20.0
25.0
1/S, liters / mmole
V, nmole / hr·mg prot.
20.0
15.0
10.0
5.0
0.5
1.0
1.5
2.0
S, mM

Table 4. The substrate specificity of the sialyltransferase reaction. Activity is expressed as the percentage of radioactivity incorporated using desialyzed α_1 acid glycoprotein as the reference value of 100%. All acceptors were present at a 2mM galactoside concentration. In the case of fetuin, desialyzed agalactofetuin, and α_1 acid glycoprotein, 2 mM refers to "potential acceptor sites". In experiments where two acceptors were used, each individual acceptor was present at a 2 mM galactoside concentration. Numbers in parenthesis indicate the numbers of experiments performed for each substrate. The variation reported is the maximum variation from the mean value.

TABLE 4: SUBSTRATE SPECIFICITY

ACCEPTOR	ACTIVITY*	
α_1 acid glycoprotein	7 ±0.5	(3)
desialyzed α_1 acid glycoprotein	100	
fetuin	0	(3)
desialyzed fetuin	78 ±8	(4)
desialyzed, agalactofetuin	0	(3)
gal β1⟶4glcNAcβ-R-BSA	33 ±4	(4)
gal β1⟶3glcNAcβ-R-BSA	0	(3)
gal β1⟶3galNAcα-R-BSA	17 ±1.3	(3)
gal β1⟶4glcNAc	26 ±3	(4)
gal β1⟶4glc	10 ±1.5	(4)
β-phenyl-galactopyranoside	0	(3)
β-thiodigalactoside	1 ±0.3	(3)
desialyzed α_1 acid glycoprotein plus gal$\beta 1 \xrightarrow{1}$4glcNAcβ-R-BSA	38 ±4.5	(3)
desialyzed α_1 acid glycoprotein plus gal$\beta 1 \xrightarrow{1}$3galNAcα-R-BSA	116 ±3	(3)

the same protein concentration as desialyzed fetuin, could not be utilized. Native α_1 acid glycoprotein showed some acceptor ability, possibly because of the presence of some galβ1→4glcNAc termini in the native molecule.

Results of competition studies are also shown in Table 4. A typical set of assay results obtained with three different membrane preparations is shown in Table 5. As indicated, galβ1→4glcNAcβ-R-BSA appears to compete with desialyzed α_1 acid glycoprotein for the active site of the enzyme, resulting in a lower activity than that obtained with desialyzed α_1 acid glycoprotein alone. However, galβ1→3galNAcα-R-BSA and desialyzed α_1 acid glycoprotein do not compete for the same enzyme, as the activities toward these acceptors are additive. These results strongly suggest that two sialyltransferases are present, one of which transfers NAN to galβ1→4glcNAc termini, and one of which transfers NAN to galβ1→3galNAc termini (conformations shown in Figure 21).

The effect of the nucleotide phosphates CMP, CDP, and CTP on sialyltransferase activity was also determined. Results (Table 6) show that CTP appears to inhibit most effectively (91%). This was followed by CDP (81%), while CMP showed the lowest inhibition (48%).

Product Separation

When the assay products were passed through columns of Sephadex G-75 as described, two peaks containing ^{14}C were obtained (Figure 16). Fractions containing large molecular weight material (peak A) were pooled and lyophilized, in order to further characterize the product.

Table 5. Competition assays performed under saturating conditions. Activity is expressed in nanomoles NAN transferred/20 min. Activity percentages represent the percentage of radioactivity incorporated using desialyzed α_1 acid glycoprotein as the reference value of 100%. Each acceptor was present at a galactoside concentration of 2 mM, totalling 4 mM in those experiments where two acceptors were used.

TABLE 5: COMPETITION ASSAYS

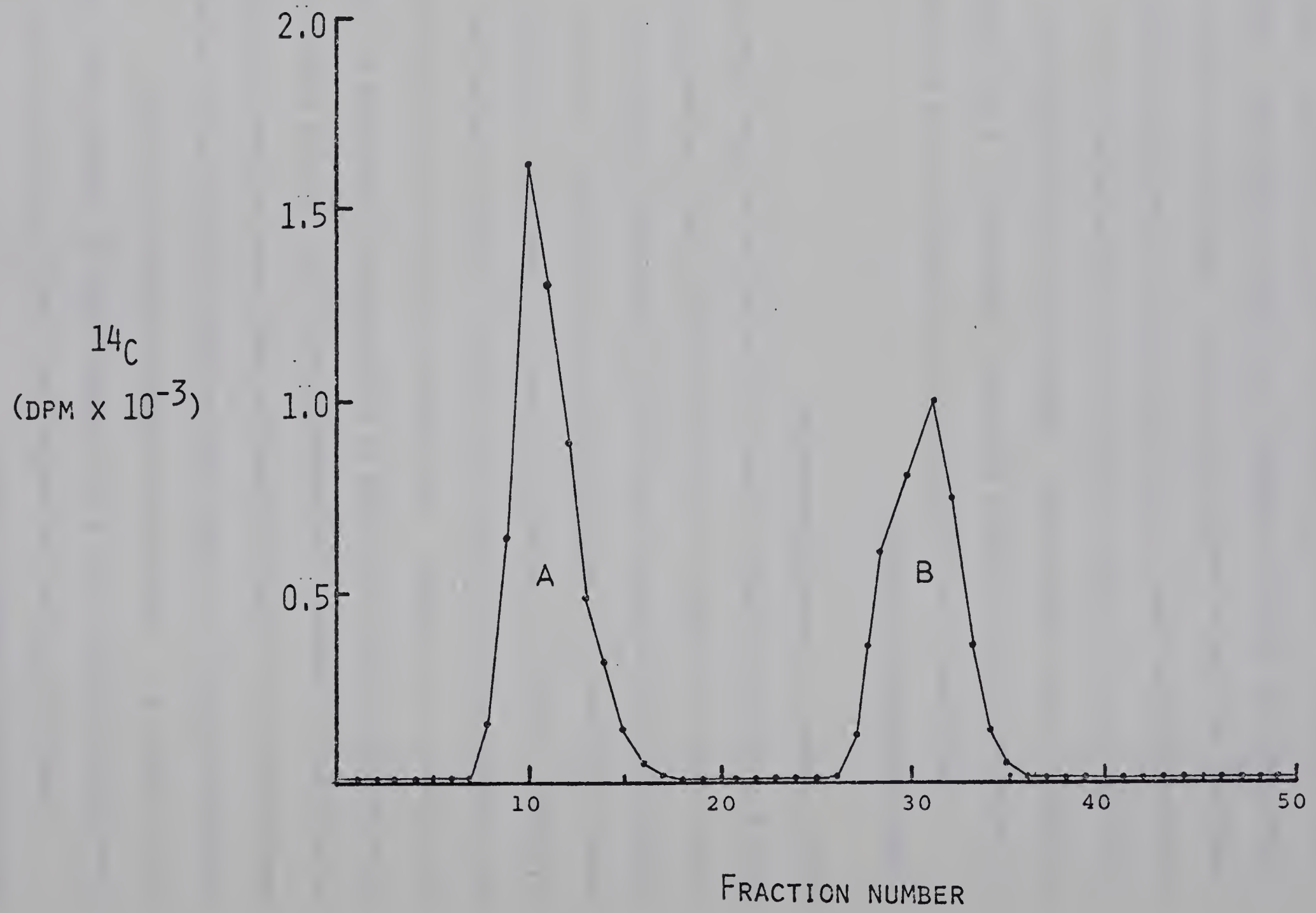
Acceptor(s)	MEMBRANE PREPARATION						
	#1		#2		#3		Average %
	Activity	%	Activity	%	Activity	%	Activity
Desialyzed α_1 acid glycoprotein	.539	100	.661	100	.445	100	100
Desialyzed α_1 acid glycoprotein plus gal β1→4glcNAcβ-R-BSA	.223	41.4	.263	39.8	.151	33.9	38.4
Desialyzed α_1 acid glycoprotein plus gal β1→3galNAcα-R-BSA	.642	119	.757	115	.512	115	116

Table 6. Inhibitory effects of cytidine phosphates on the sialyltransferase activity. The complete system contained 40-150 μg membrane protein, 2mM galactoside termini on desialyzed α_1 acid glycoprotein, 0.6 mM ^{14}C-CMP-NAN (1.67 mCi/mmole), 1% Triton X-100, and cacodylate-HCl buffer (50 mM), pH 5.5. The mixtures were incubated at 30°C for 20 minutes. Each value represents the average of two experiments in which variation was not more than ±7.3% of the mean value.

TABLE 6: ENZYME INHIBITION STUDIES

SYSTEM	% MAXIMAL ACTIVITY
Complete System	100
plus 1 mM CMP	52
plus 1 mM CDP	19
plus 1 mM CTP	9

Figure 16. Separation of ^{14}C-labelled, high molecular weight product (peak A) from ^{14}C-CMP-NAN and ^{14}C-NAN (peak B), using Sephadex G-75.


^{14}C
(DPM X 10^{-3})
2.0
1.5
1.0
0.5
A
B
10
20
30
40
50
FRACTION NUMBER

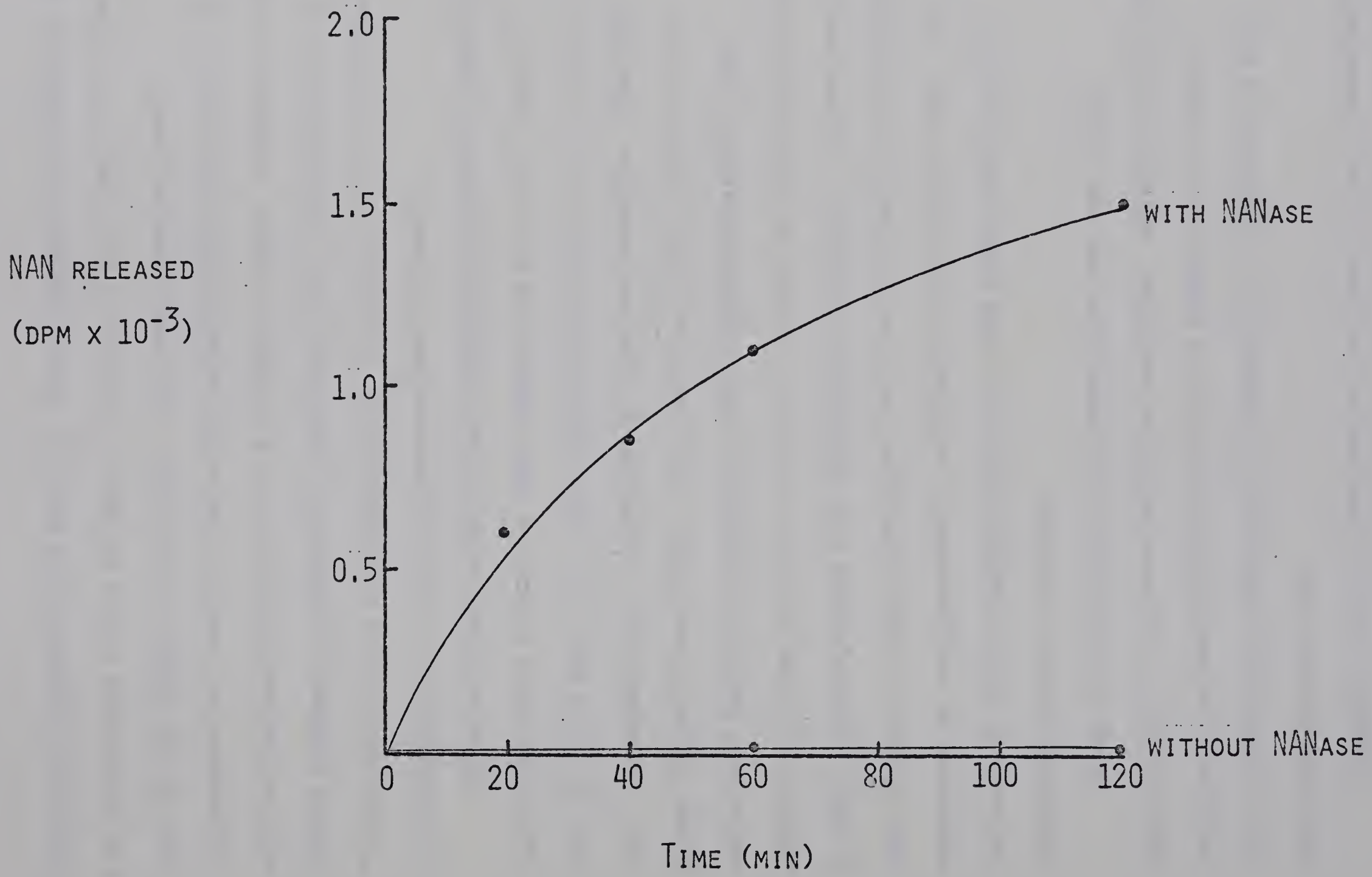
Neuraminidase Treatment of Product

When the ^{14}C-labelled product was treated with neuraminidase from *Vibrio cholerae*, 79% of the ^{14}C-NAN could be released after two hours (Figure 17). Controls run without neuraminidase showed negligible release of ^{14}C-NAN from the large molecular weight material. Neuraminidase from *Vibrio cholerae* has been shown to cleave sialosides of only the α linkage at the anomeric carbon, C-2 (Yu and Ledeen, 1969). This strongly suggests that NAN is transferred to desialyzed α_1 acid glycoprotein in α linkage. Since galactose is the terminal sugar in the acceptor molecules tested, the linkage is likely to galactose, although a linkage to N-acetylglucosamine is possible. The latter sugar is present in a subterminal position to galactose, and the possibility that galactose may be only a requirement in order for sialic acid to be added to N-acetylglucosamine has to be considered. This possibility is small, however, since NAN has not been found in nature linked to N-acetylglucosamine.

Smith Degradation of Product

Desialyzed α_1 acid glycoprotein, ^{3}H-labelled at the C-6 position of galactose by the procedure described, contained 3.1×10^7 dpm per milligram of this glycoprotein. Desialyzed α_1 acid glycoprotein (0.72 mg, 2.23×10^7 dpm) was used as the acceptor molecule in a sialyltransferase assay in which six volumes of the reaction mixture described for routine assays was utilized. Following the reaction, the mixture was run through a Sephadex G-75 column as described. Aliquots were counted for ^{3}H and ^{14}C; results are shown in Figure 18. Fractions 7-12 were pooled, brought to a volume of 2 ml as described and a 10 µl

Figure 17. Release of ^{14}C-NAN from sialylated α_1 acid glycoprotein using neuraminidase. Each reaction mixture initially contained 1940 dpm of ^{14}C-NAN linked to desialyzed α_1 acid glycoprotein. Release of NAN with time was measured as described in the text. Each data point represents the average of duplicate experiments in which variation was never more than ±3.5% of the mean value.


NAN RELEASED
(DPM X 10^{-3})
2.0
1.5
1.0
0.5
WITH NANASE
WITHOUT NANASE
0
20
40
60
80
100
120
TIME (MIN)

aliquot was counted for ^{3}H and ^{14}C. Total radioactivity present in the 2 ml mixture was 1.22×10^{7} dpm of ^{3}H and 1.76×10^{4} dpm of ^{14}C. The ratio of dpm ^{3}H/dpm ^{14}C was 693/1. The double-labelled product (represented by A and A', Figure 18) contains 55% of the original ^{3}H added to the assay mixture (2.23×10^{7} dpm). Peak B, composed of some unknown component, contains ^{3}H, but little ^{14}C. The large peak C is ^{14}C-labelled CMP-NAN and possibly NAN. The amount of NAN transferred to the desialyzed α_1 acid glycoprotein was 4.73 nmoles.

After periodate oxidation and subsequent borohydride reduction, the radioactivity present in this compound was 9.68×10^{3} dpm of ^{14}C and 6.81×10^{6} dpm of ^{3}H. This indicates that 56% of the ^{3}H and 55% of the ^{14}C remained after the oxidation/reduction procedure. The ratio of dpm ^{3}H/dpm ^{14}C was 704/1. This indicated that a portion of the sialic acid (which was labelled at C-4) remained attached to the glycoprotein after periodate oxidation, and that no differential loss of label occurred during this experimental procedure. The decrease of both isotopes in equal proportions could be explained by the possible adsorption of the glycoprotein to glassware and dialysis bags, since the ratio of $^{3}H/^{14}C$ remained nearly the same before and after the oxidation/reduction procedure.

After acid hydrolysis, the hydrolysate was run through Amberlite IR-120 and Dowex AG2-X8 as described. Aliquots of fractions coming off the Dowex AG2-X8 showed no ^{14}C present, but a large ^{3}H peak was recovered (fractions 2-6, Figure 19). Fractions 2-6 were pooled and concentrated.

Products present in the pooled fractions 2-6 were chromatographed

Figure 18. Separation of large molecular weight product, double-labelled, from small molecular weight compounds on Sephadex G-75. Closed circles represent ^{14}C, open circles represent ^{3}H.

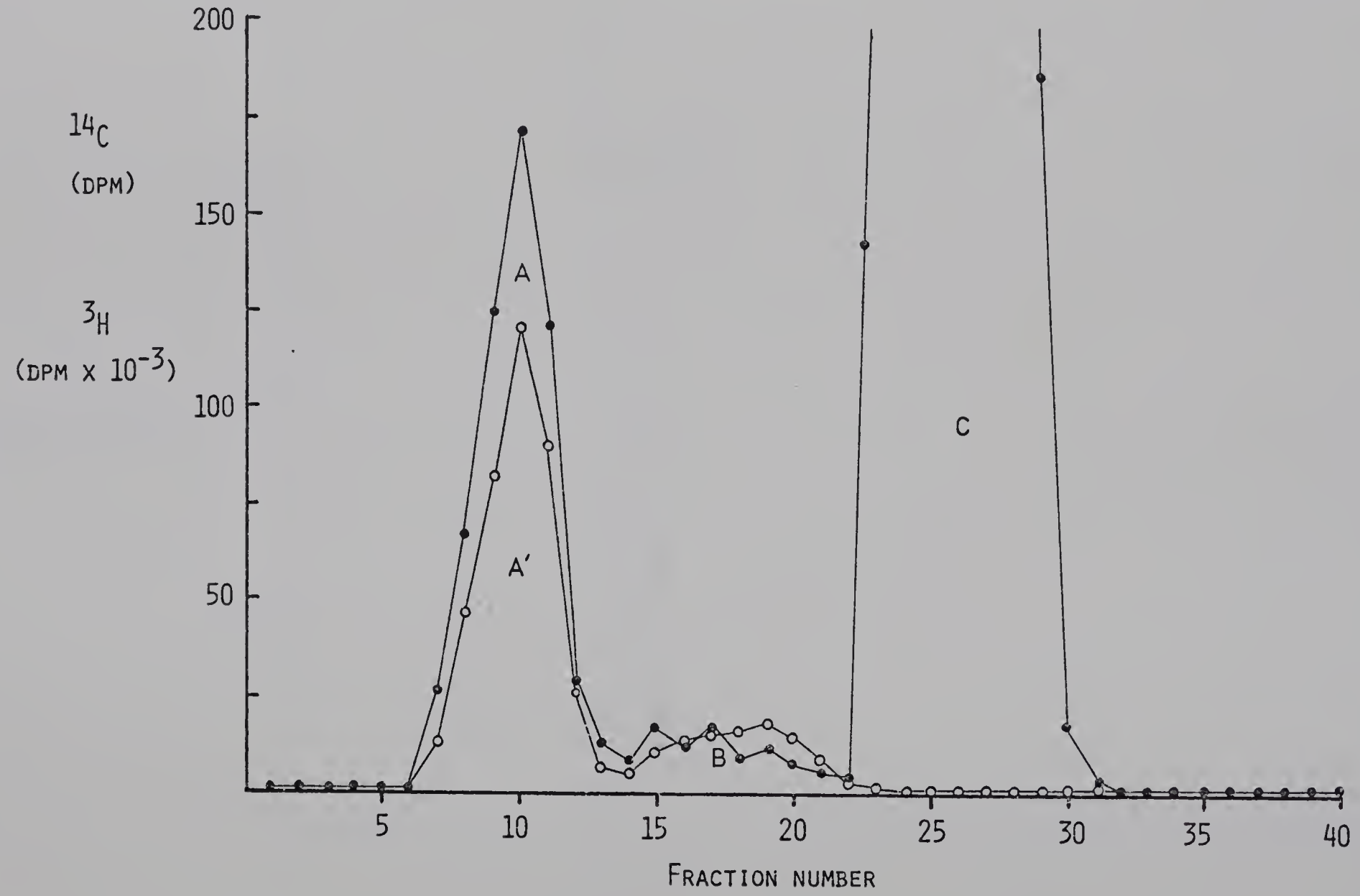
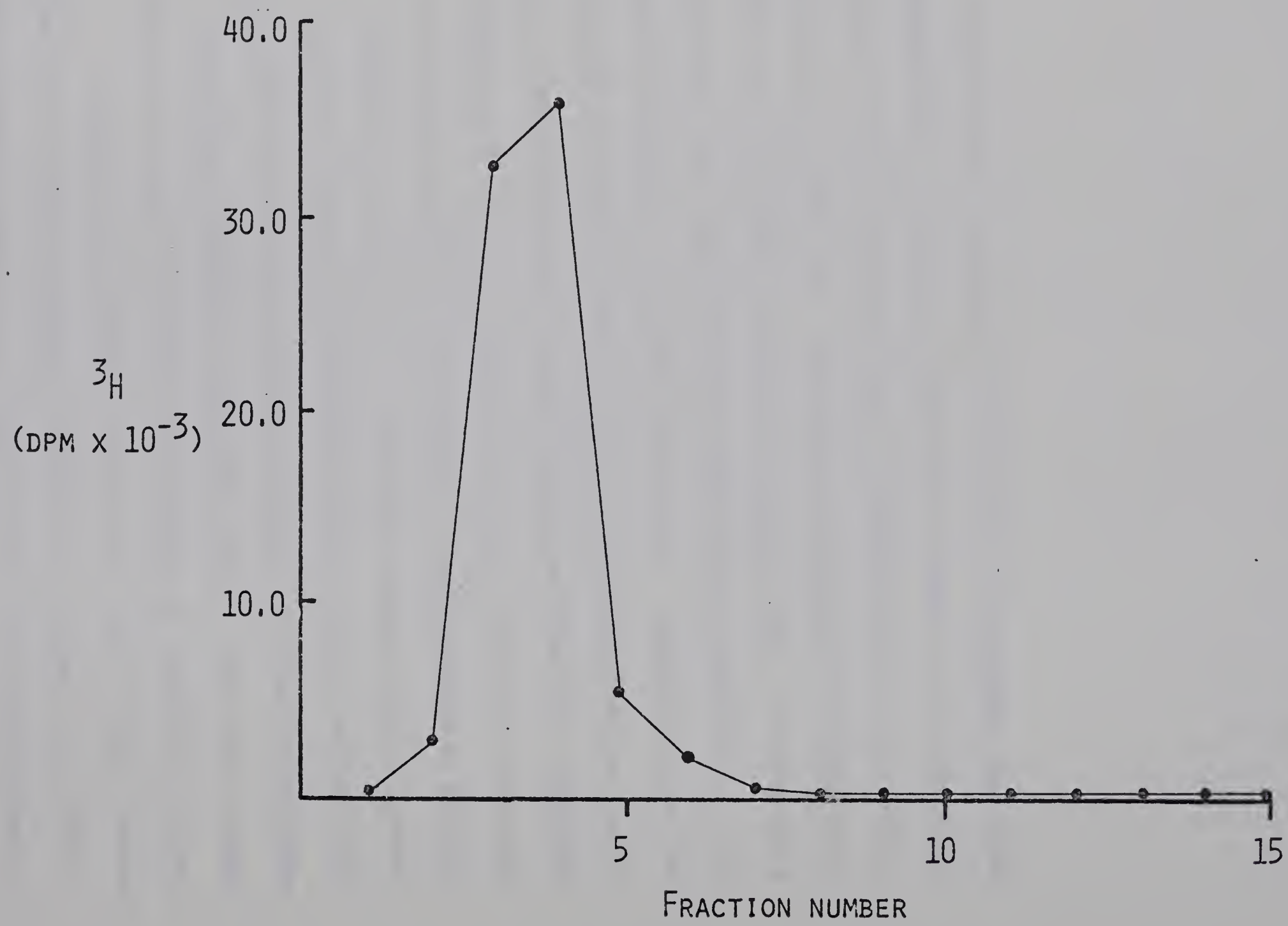

200
150
100
50
^{14}C
(DPM)
^{3}H
(DPM x 10^{-3})
A
A'
B
C
5
10
15
20
25
30
35
40
FRACTION NUMBER

Figure 19. Smith degradation: Elution pattern of ^{3}H-labelled compounds off a Dowex AG2-X8 column following acid hydrolysis.


40.0
30.0
20.0
10.0
^3H
(DPM X 10^{-3})
5
10
15
FRACTION NUMBER

as described, and the tritium profiles on the chromatograms are shown in Figure 20. Control 1, in which no CMP-NAN was added to the assay mixture, shows a single peak which co-migrates with glycerol. Control 2, in which no CMP-NAN and no enzyme was added to the assay mixture, also shows a single peak which co-migrates with glycerol. The experimental sample, in which NAN had been linked to the glycoprotein, also displays a large ^{3}H peak co-migrating with glycerol (peak B). However, it also contains minor peaks (A, C, D); no ^{14}C could be detected on chromatograms. The minor peak A migrates similarly to galactose, but peaks C and D do not migrate with any of the expected standards. The small peak between peaks B and C may be due to combined amounts of the radioactivity present in peaks B and C, or may represent yet another compound. In different experiments the degree of concentration of the product in the sample chromatographed varied (200-400 µl final volume). Hence, different amounts of radioactivity were applied at the origin on different chromatograms. The radioactivity profiles (Figure 20) have been adjusted so that the glycerol peaks on different chromatograms contained the same amount of tritium. The chromatograms were then compared as if they had equal amounts of radioactivity.

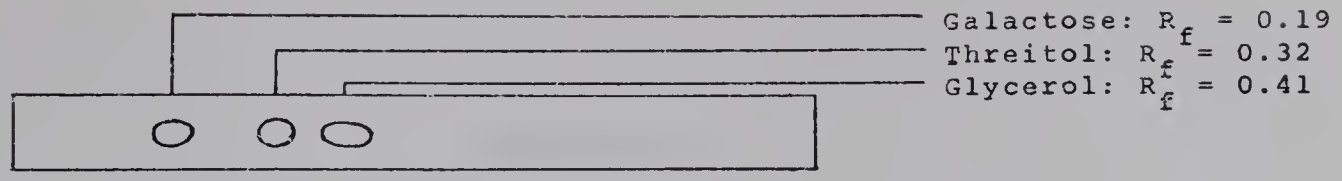
Figure 20. Paper chromatography of the tritiated products of the Smith degradation procedure. Results were adjusted so that equal amounts of tritium are present under the large glycerol peaks on each radioactivity profile.

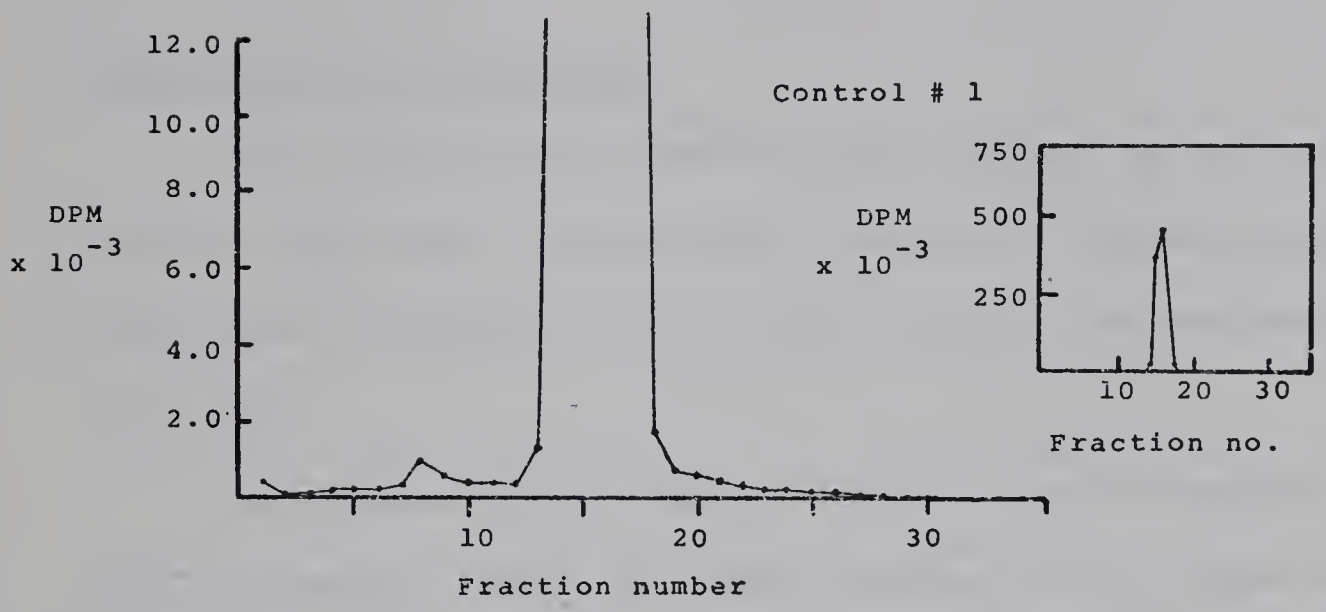
Control # 1. Tritiated products obtained when the sialyltransferase assay was performed in the absence of CMP-NAN.

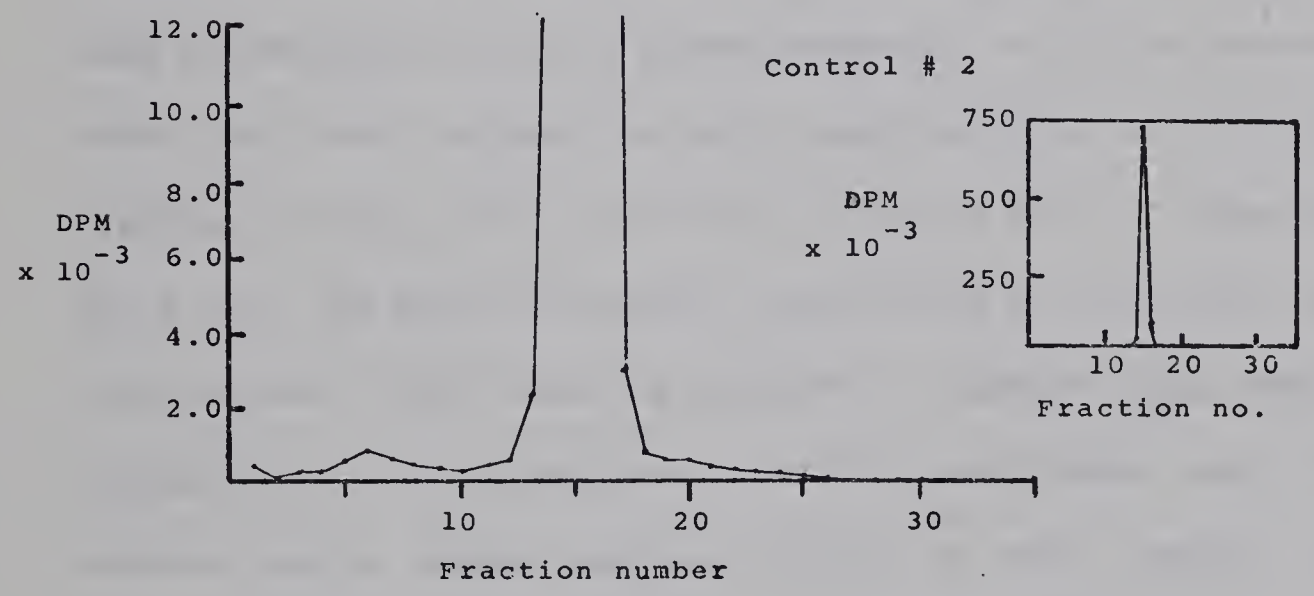
Control # 2. Tritiated products obtained when the sialyltransferase assay was performed in the absence of both CMP-NAN and plasma membranes.

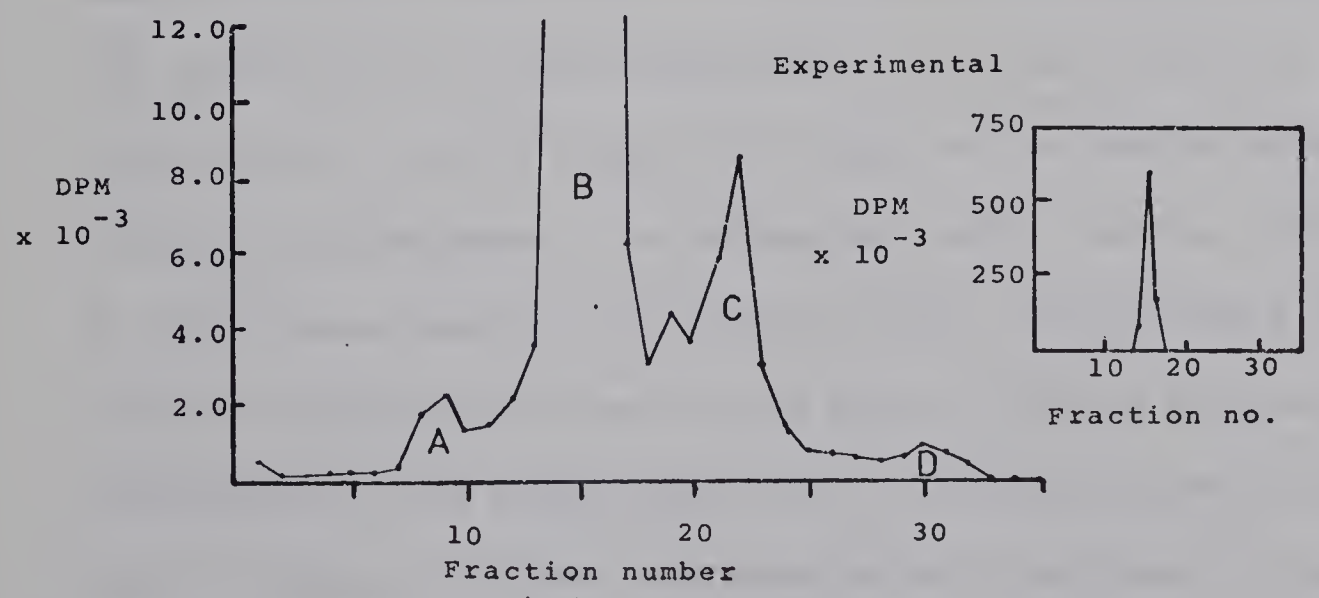
Experimental. Tritiated products obtained when the sialyltransferase assay was performed in the presence of CMP-NAN and plasma membranes.

The migration of standards is shown at the top of the figure. The point of application of standards was to the left.


Galactose: R_f = 0.19
Threitol: R_f = 0.32
Glycerol: R_f = 0.41


Control # 1
DPM x 10^{-3}
12.0
10.0
8.0
6.0
4.0
2.0
10
20
30
Fraction number
DPM x 10^{-3}
750
500
250
10
20
30
Fraction no.


Control # 2
DPM x 10^{-3}
12.0
10.0
8.0
6.0
4.0
2.0
10
20
30
Fraction number
DPM x 10^{-3}
750
500
250
10
20
30
Fraction no.


Experimental
DPM x 10^{-3}
12.0
10.0
8.0
6.0
4.0
2.0
A
B
C
D
10
20
30
Fraction number
DPM x 10^{-3}
750
500
250
10
20
30
Fraction no.

DISCUSSION

Plasma Membrane Preparation

The results of the enzyme marker studies, as well as the electron microscopy, indicate that the galβ1→4glcNAc specific sialyltransferase is enriched in the plasma membrane preparations obtained in this study.

Two plasma membrane marker enzymes, a Mg^{++}-dependent ATPase and a Mg^{++}-dependent CTPase, are found purified in the plasma membrane preparation used in this work. Sanford and Rosenberg (1972), who have used an entirely different plasma membrane isolation procedure, have shown that these enzymes are only found purified in the plasma membrane fraction of cells from the 15-day old chick liver. These authors also found that the above-mentioned enzymes were not purified in microsomal preparations, which would be expected to contain Golgi membrane fragments as well as portions of smooth endoplasmic reticulum. Other enzymes used as plasma membrane markers in other tissues, such as ouabain-sensitive ATPase or 5' nucleotidase (Zalik and Cook, 1976) have not appeared in the plasma membranes of the chick liver at the developmental stages studied herein, and could not be used as markers. No succinic dehydrogenase, a mitochondrial marker enzyme, could be detected in this plasma membrane preparation (Zalik, unpublished results). The results mentioned above as well as direct electron microscopical examination of the preparations used in the experiments reported in this thesis indicate that they are substantially enriched in plasma membranes. The 8.6-fold purification of the sialyltransferase in this preparation

closely matches the purification of the CTPase (8.5-fold) and ATPase (6.8-fold), and strongly suggests this glycosyltransferase is present in plasma membranes. Since the preparations of Sanford and Rosenberg were from 15-day old embryonic livers, and these experiments were performed on 13-day old embryos, it is possible that their purifications of 13.5-fold for CTPase and 15.6-fold for ATPase are higher because these enzymes may increase in quantity in the plasma membranes of the chick liver cells during development. It is known that changes occur in the embryonic liver cells during development (Romanoff, 1960). The parenchymal cells increase in size between 12 and 15 days, and lipid-containing vesicles in their cytoplasm become more numerous. Also, the Golgi apparatus of parenchymal cells becomes more extensive, and endothelial cells begin to differentiate to become granulocytes. However, it is also possible that the differences in the abundance of the above-mentioned enzymes in the membrane preparations are due to the loss of enzymatic activity during the preparation procedure used in this report, which requires the use of solutions of relatively high pH (9.6).

In electron micrographs, no rough endoplasmic reticulum could be seen. The only other sources of smooth membrane contamination in the plasma membrane preparation could be the Golgi apparatus or the smooth endoplasmic reticulum. The Golgi apparatus, if intact, usually appears as flattened sacs in electron micrographs (Whaley et al., 1975). If it ruptures, however, fragments of smooth membranes result. Normally, fragments of Golgi membranes and smooth endoplasmic reticulum rearrange to form relatively small vesicles which would not sediment to an appreciable extent after centrifugation at 9,000 x g for one hour.

These small membrane vesicles usually sediment in the microsomal, or "post-mitochondrial" pellet, obtained after centrifugation at 100,000 x g for 1-2 hours (Beaufay and Amar-Costesec, 1976). In the present plasma membrane isolation procedure, plasma membranes form much larger "ghosts," which sediment after centrifugation at 9,000 x g for one hour. The possibility of larger Golgi fragments being present in the preparation, however, cannot be excluded. In addition, it was noted that no lysosomes could be seen in the plasma membrane preparation, when sections were viewed under the electron microscope.

The marker enzymes used to identify the Golgi apparatus are typically the glycosyltransferases (Whaley et al., 1975). In this instance, however, the glycosyltransferases cannot be used as Golgi markers, since they could be present in both Golgi membranes and plasma membranes. In fact, any Golgi apparatus marker enzyme, if found in a plasma membrane preparation, would not necessarily mean that Golgi membranes are contaminating the preparation. Cellular plasma membranes contain membrane material derived directly from the Golgi apparatus via fusion of secretory or other types of Golgi-derived vesicles with plasma membranes (Whaley et al., 1975; also see the Introduction). The presence of a Golgi enzyme in a plasma membrane preparation would not necessarily indicate the presence of membranes of the Golgi complex. The only way to definitely show that Golgi membranes are absent from this preparation would be to purify Golgi apparati, and find marker enzymes present in the Golgi preparation which were absent in purified plasma membrane preparations. Since Golgi apparati were not purified in this report, the possibility that the sialyltransferase activity may be

in part due to Golgi contamination cannot be completely ruled out.

It has been suggested that plasma membrane glycosyltransferases may be asymmetrically located in the plasma membrane with their active sites oriented to the exterior, and that they may be involved in cellular adhesion. The mechanism mentioned before of externalization of membrane components derived from the Golgi apparatus would account for this possibility. However, definite proof of cell-surface enzyme activity is very difficult to obtain. Assays using intact cells are difficult to interpret, as discussed in the Introduction (Keenan and Morré, 1975; and Deppert and Walter, 1978). In these experiments, labelled nucleotide-sugar donor added to "intact" cell suspensions could theoretically be degraded outside of the cell, and the sugar could be transported and utilized internally, to subsequently appear in cell-surface polysaccharides. In addition, the nucleotide-sugars could be taken inside the cell by pinocytosis, and utilized internally after degradation to smaller components.

The labelled sugar could also be transferred directly to cell-surface carbohydrates, either by plasma membrane glycosyltransferases or by glycosyltransferases released into the medium due to cell lysis. In addition, cytoplasmic glycoproteins, which may in turn act as endogenous acceptors, could be released due to cell lysis into the surrounding medium; the incorporation of a labelled sugar into a high molecular weight acceptor could represent an artifactual cell-surface associated activity. Hence, the incubation of "intact" cells with nucleotide-sugars yields results which are associated with a number of ambiguities, and must be interpreted with a degree of caution, as it is difficult to

discern just where synthesis is occurring.

Despite the frailties associated with experiments designed to detect cell-surface glycosyltransferases in intact cells, preliminary experiments were performed by this author, using cell suspensions of embryonic livers. Preliminary data showed that sialyltransferase activity was released into the surrounding medium, making interpretations of the experiments difficult. Even if cells were washed five times, leakage of the enzyme into the medium was observed. As yet, no definite statements can be made as to the orientation of the sialyltransferase within plasma membranes. The experiments reported herein indicate the presence of the enzyme in the plasma membrane preparations of embryonic liver cells, and shed light on the work of Arnold et al. (1973, 1976). These authors, using cell suspensions of embryonic liver, reported the presence of several glycosyltransferases capable of transferring sugars to endogenous acceptors. They claimed that these transferases were cell-surface associated, under the assumption that glycosyltransferases were not released into the surrounding medium from whole "intact" cells. In the present work, it was found that sialyltransferase activity was released into the medium. The claim of the above-mentioned authors that these enzymes are surface located should be viewed with caution, since controls for release of transferases were not performed by these authors. Nevertheless, other reports on cell-surface sialyltransferases (Bernacki and Porter, 1978; Yogeeswaren et al., 1974; Lloyd and Cook, 1974; and Cervén, 1977) provide convincing evidence for the locale of the active site of glycosyltransferases on the outer face of the plasma membrane. A growing body of evidence documents the presence of these

enzymes in purified plasma membrane preparations, and the functions of particular cell-surface glycosyltransferases are only beginning to be examined.

The Sialyltransferases

Competition studies have shown that at least two sialyltransferases are present in the plasma membranes of the embryonic chick liver cells. One of these transferases is specific for galβ1→4glcNAcβ-R structures, and another is specific for galβ1→3galNAcα-R structures (structures shown in Figure 21). Of the other saccharides tested, the only structure that could be utilized was galβ1→4glc (lactose), which was glycosylated to a lesser extent, possibly by the same enzyme which acts on galβ1→4glcNAc terminal structures. Other galactosides (β-phenylgalactopyranoside, galβ1→3glcNAcβ-R, and β-thiodigalactoside) were not effective acceptors of sialic acid.

Studies on the sialyltransferase activity specific for galβ1→4glcNAc-R structures showed that enzyme activity was nearly linear with time for a 20-minute period, hence assays were performed for only 20 minutes. The enzyme had a pH optimum of 5.5, and a temperature optimum of 30° C. Boiling the solution containing the enzyme for one minute destroyed most of the enzyme activity, which would be likely to occur if the tertiary structure of the enzyme was altered at high temperatures. Optimal pH values reported for different sialyltransferases have been found to vary. Other cases where relatively low pH optima have been reported include Keller et al. (1979) (pH 5.5) and Schachter et al. (1970) (pH 5.7).

Triton X-100, a non-ionic detergent, was found to increase by approximately tenfold the activity of the sialyltransferase. Many of the membrane-bound glycosyltransferases that have been studied have been found to be differentially affected by detergents. Some are activated by detergents (Schachter et al., 1970; Kim et al., 1971), while others lose activity (Madappally et al., 1976). Detergents such as Triton X-100 are known to solubilize membranes by interfering with the hydrophobic interactions of phospholipids in the lipid bilayer of membranes (Singer, 1971). As a consequence, micelles, consisting of protein, phospholipid, and detergent molecules, result. Enzymes which require specific lipids, or which require the structural integrity of the membrane for their function, often lose activity in the presence of detergents. Other enzymes may tolerate detergent treatment without alterations in their tertiary structure, or may be modulated in their function by virtue of their interaction with specific components within an organized membrane. These enzymes could conceivably increase in activity upon detergent treatment. The sialyltransferase, being present in a plasma membrane preparation, was found to have an increased activity upon detergent treatment; the result was not too surprising, since many glycosyltransferases have been found to be activated by detergents. The enzyme could possibly be located on the interior surface of some of the vesicles present in the plasma membrane preparation, thereby restricting its access to the substrates during the assay without detergent. It is also possible that the active site of the enzyme is sterically hindered within the organized bilayer of the plasma membrane, preventing maximal interaction of the enzyme with its substrates.

The effects of divalent cations on the activity of the enzyme indicate that Ca^{++} is an inhibitor of the enzyme. At a concentration of 10 mM, Ca^{++} inhibited the enzyme by about 50%. It is not known whether Ca^{++} exerts its effect by interacting with the enzyme at the active site, or in an allosteric manner, or whether Ca^{++} interacts with one of the substrates. CMP-NAN may interact with Ca^{++}, since it contains negative charges on the phosphate and on the carboxyl group of the sialic acid. Mn^{++} and Mg^{++} may not interact as strongly with CMP-NAN, because of differences in ionic size. Mn^{++} inhibits the enzyme to a lesser extent, and Mg^{++} appears to have little inhibitory effect. Jaques et al. (1977) have shown that Ca^{++} interacts with the β anomer of N-acetylneuraminic acid, producing changes in the chemical shifts of the ^{13}C NMR spectrum. Mg^{++} did not appreciably interact with NAN. It is possible, then, that Ca^{++} may interact with CMP-NAN, producing the inhibition observed in this report. It is of interest that Mn^{++}, in particular, does not increase the activity of the enzyme, as Mn^{++} is known to be necessary for the activity of a number of glycosyltransferases (Schachter and Rodén, 1973).

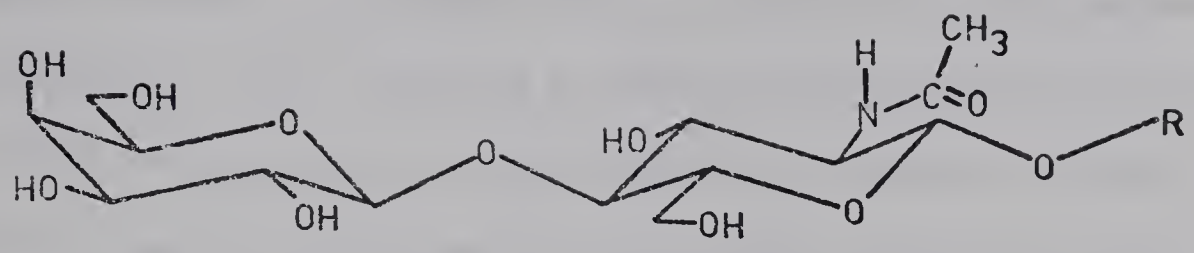
CTP, CDP, and CMP were also inhibitors of the sialyltransferase. CTP was the best inhibitor, with CDP and CMP inhibiting to successively lesser extents. It would appear, then, that these nucleotides are capable of interfering with the reaction catalyzed by the sialyltransferase, possibly by occupying the active site of the enzyme where CMP-NAN would normally interact. This effect has been noted with other sialyltransferases (Paulson et al., 1977a; Rearick et al., 1979), and CDP linked to columns has actually been used as an affinity adsorbent

to purify sialyltransferases (Paulson et al., 1977b; Sadler et al., 1979).

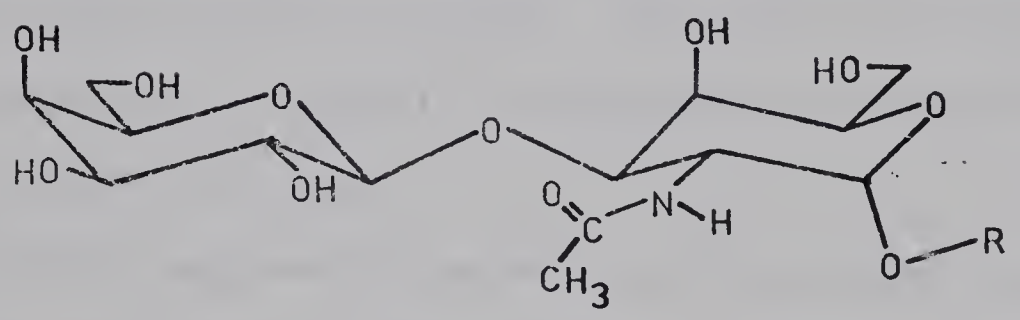
For specificity studies, assays were performed under optimal conditions for enzyme activity; saturating amounts of both substrates were utilized. Studies of the specificity of the enzyme indicate that galβ1→4glcNAc is a necessary portion of the acceptor molecule, since sialic acid was added to all structures containing this disaccharide moiety. The N-acetamido group on glucose would appear to be important in this reaction, since lactose (galβ1→4glc) was not utilized as effectively as galβ1→4glcNAc. In addition, the composition of the aglycone group linked to the galβ1→4glcNAc moiety of the acceptor appears to affect the activity of this particular transferase. The aglycone group of the chemically synthesized "glycoprotein," which consists of an aliphatic carbon chain linked to bovine serum albumin, was not as effective an acceptor as desialyzed α_1 acid glycoprotein or desialyzed fetuin, in which the aglycone group consists of an oligosaccharide linked to protein. Differences were observed between these "natural" glycoprotein acceptors as well, since desialyzed fetuin was not as effective an acceptor as desialyzed α_1 acid glycoprotein. These results indicate that the enzyme recognizes more than just the terminal galβ1→4glcNAc of the acceptor molecule, and may require other subterminal structures for full activity. When the terminal galactoside is either masked by sialic acid, as in native fetuin and α_1 acid glycoprotein, or when the galactose is removed from the acceptor, as in desialyzed, agalactofetuin, the glycoproteins do not serve as acceptors for the sialyltransferase.

As shown in Figure 21, the structures of galβ1→4glcNAc and galβ1→3galNAc are significantly different, and the different specificities exhibited by the two enzymes for these substrates is understandable when the conformations of the acceptors are examined. If the galactose groups of these molecules were superimposed, the aglycone groups of the galactoside would not have a single functional group which is superimposable. This type of glycosyltransferase specificity is not unusual, but quite common in studies where the specificity of these enzymes has been examined. Hudgin and Schachter (1971) reported a sialyltransferase from porcine liver capable of transferring sialic acid to terminal galβ1→4glcNAc structures of glycoproteins, as well as to the disaccharide alone. Paulson et al. (1977a) purified a sialyltransferase from bovine colostrum and showed that it was capable of transferring NAN specifically to galβ1→4glcNAcβ-R structures. This enzyme produced NANα2→6galβ1→4glcNAcβ-R structures. Like the sialyltransferase in this report, the enzyme also appears to have a requirement for more than just the disaccharide terminus of a glycoprotein, because NAN was transferred to the reducing disaccharide, galβ1→4glcNAc at a rate approximately one-tenth of the rate obtained using several glycoprotein acceptors with galβ1→4glcNAcβ-oligosaccharide-protein structures. This same group of researchers have recently purified to homogeneity a sialyltransferase from porcine submaxillary glands which specifically utilizes acceptors which have a galβ1→3galNAc terminus. The linkage was characterized as α2→3 to galactose (Rearick et al., 1979). The same group have also purified a fucosyltransferase which apparently adds L-fucose to the glcNAc in galβ1→4glcNAc structures

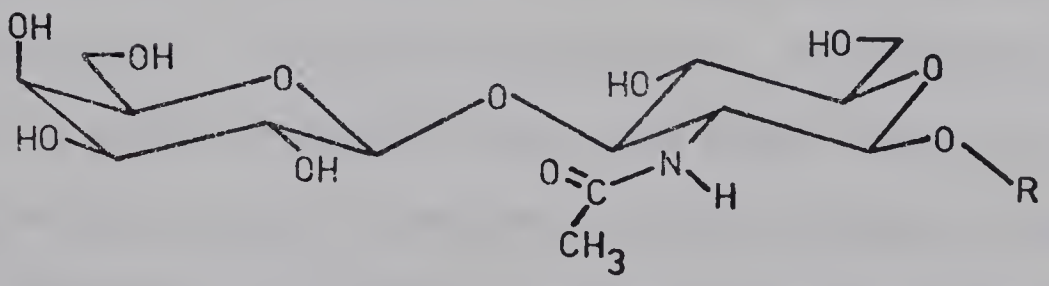
Figure 21. Conformational drawings of three disaccharide structures used as acceptors for the sialyltransferases reported herein.



βgal 1→4βglcNAc 1→R



βgal 1→3∝galNAc 1→R



βgal 1→3βglcNAc 1→R

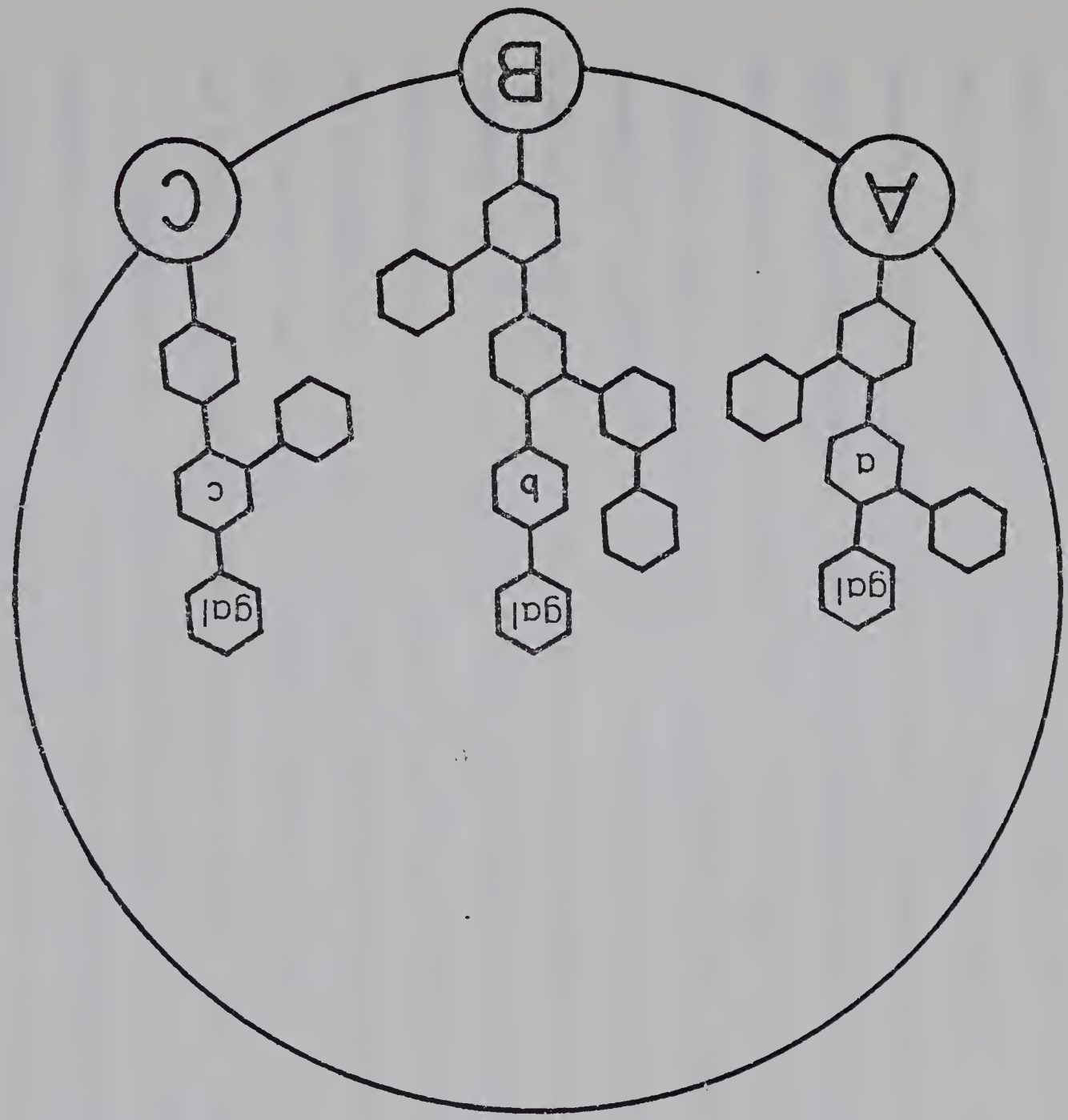
(Prieels et al., 1977; Paulson et al., 1978; Prieels and Beyer, 1979). Fucose, in this instance, is linked α1→3 to the N-acetylglucosamine. Jabbal and Schachter (1971) reported a similar fucosyltransferase in pork liver which was specific for galβ1→4glcNAc structures; these authors did not, however, characterize the linkage catalyzed by the enzyme. Carne and Watkins (1977) have purified a "B" blood group galactosyltransferase which specifically transfers galactose α1→3 to the subterminal galactose of H-active glycoproteins, glycolipids, and smaller saccharides (all of which have a fucα1→2gal terminus). A blood group "A" N-acetylgalactosaminyltransferase has been purified from porcine submaxillary glands (Schwyzer and Hill, 1977) which transfers N-acetylgalactosamine specifically to the galactose of αfuc1→2gal termini of H-active substances.

The studies mentioned above indicate that a number of glycosyltransferases which synthesize the peripheral portions of glycoproteins are specific for at least the terminal disaccharide of an acceptor molecule. Since this specificity appears to be so widespread among these enzymes, it may be more than just an interesting observation. In fact, such specificities may be a necessity in many cells which simultaneously produce several carbohydrate structures. As different polysaccharides are being synthesized within the Golgi apparatus, the same monosaccharide might appear at the non-reducing terminus of several different heterosaccharides. If different sugars, or the same sugar in different linkages, are to be attached to the same terminal monosaccharide on different growing polysaccharide chains, then the glycosyltransferases involved must recognize structures which are subterminal to the terminal

monosaccharide (see Figure 22). In this way, correct synthesis of all the glycoconjugates can be achieved simultaneously within the Golgi apparatus. To clarify this point, consider the biosynthesis of the glycoprotein fetuin (structure shown in Figure 6). In order for sialic acid to be transferred in the proper linkage to galactose and N-acetyl-galactosamine residues on the glycoprotein during its synthesis, at least four sialyltransferases with different specificities would be required. One of these would add NAN α2→6 to galNAc, and should not utilize galactosides as substrates, to avoid linking NAN randomly α2→6 to the galactosides. Another sialyltransferase would need to recognize the galβ1→3galNAc structure present on the molecule, and specifically add NAN α2→3 to it. The other two sialyltransferases would need to be specific for particular galβ1→4glcNAcβ-R portions of the complex carbohydrate, to produce the proper NAN α2→3 or α2→6 linkage to the proper galβ1→4glcNAc terminus. If these sialyltransferases could add to any β-galactoside on fetuin, then NAN would be found randomly linked α2→6 and α2→3 to all of the galactosides of this glycoprotein, which is not the case found in nature. This glycoprotein is very interesting when viewed from a biosynthetic viewpoint, and characterization of its mode of synthesis would be of general interest in the study of polysaccharide synthesis.

It should be mentioned that one glycosyltransferase that will transfer to a monosaccharide acceptor has been described. The galacto-syltransferase in milk which synthesizes lactose transfers galactose normally to N-acetylglucosamine. In the presence of lactalbumin, a modulating protein present within the mammary gland during lactation,

Figure 22. A schematic representation of three glycoconjugates (A,B,C) being synthesized in the Golgi apparatus. Each has a terminal non-reducing galactose. Subterminal sugars (a,b,c) can either be different monosaccharides or the same monosaccharide linked in a different manner to galactose. If different sugars are to be transferred to the galactose in different linkages (for instance, NAN α2→3gal on A, fucose α1→3gal on B, and NAN α2→6gal on C), then, in order to consistently produce the proper product, the glycosyltransferases must recognize more than just the terminal galactose. They may be specific for the terminal disaccharide (gal→a, gal→b, or gal→c), as well as other subterminal structures in the glyconjugate.


A
B
C
gal
gal
gal
d
b
c

the enzyme catalyzes the transfer of galactose to glucose, in a β1→4 linkage (Brew et al., 1968; Schachter and Rodén, 1973).

Livers from a number of sources have been examined for the presence of sialyltransferases. Hudgin and Schachter (1972) discovered in porcine, rat, bovine, and human livers sialyltransferases capable of synthesizing sialyllactose, in which NAN was linked α2→3 and α2→6 to the galactose of lactose. In addition, in porcine livers, a sialyl-transferase which transfers NAN specifically to glycoproteins with a galβ1→4glcNAcβ-R terminus, and to the disaccharide galβ1→4glcNAc has been studied by the same authors (1971). In this case, however, the linkage of NAN to galactose was not characterized. This enzyme resembles the embryonic chicken liver transferase in specificity, but does not appear to be the same enzyme, as its pH optimum was 7.0, compared to 5.5 for the embryonic chick liver enzyme. In addition, the kinetic constants for the two enzymes were also considerably different. The K_m values were 2.8 mM for terminal galactoside acceptors of α_1 acid glycoprotein, and 0.19 mM for CMP-NAN, compared to values of 0.18 mM and 0.017 mM, respectively, obtained for the chicken liver enzyme.

Schachter et al. (1970) and Van den Eijnden et al. (1977) have reported a sialyltransferase present in rat liver microsomes which catalyzed the transfer of sialic acid to desialyzed α_1 acid glycoprotein only in α2→6 linkage. This enzyme is similar to the one reported here, in that it is activated by Triton X-100, and has a pH optimum of 5.7. However, kinetic constants were not reported, making it difficult to claim that these are the same enzyme. Hudgin and Schachter (1972) have also reported sialyltransferase activity in rat livers which resulted

in the formation of α2→3 and α2→6 linkages to lactose. Sialyltransferase activities have been examined in fetal mouse livers (Madappally et al., 1976) and in human livers (Alhadeff et al., 1977) using exogenous acceptors (desialyzed fetuin, and desialyzed mucins), but specificity studies or linkage analyses were not performed.

Linkage Characterization

In nature, NAN is linked α2→3, α2→4, and α2→6 to galactose, α2→6 to N-acetylgalactosamine, and α2→8 to NAN (reviewed by Tuppy and Gottschalk, 1972). Due to the possibility that different sialyltransferases can link NAN to various positions on galactoside acceptors, it is important to examine the type of linkage catalyzed by a particular sialyltransferase.

Neuraminidase from *Vibrio cholerae* has been shown to cleave only α sialosides (Yu and Ledeen, 1969). Previous work using synthetic β sialosides were not susceptible to cleavage with neuraminidase. In the experiments reported herein, *Vibrio cholerae* neuraminidase cleaved, within 2 hours, nearly 80% of the NAN from the product of the sialyltransferase reaction. These results strongly suggest that sialic acid is linked in an α configuration to galactose. This is in accord with all known sialoside linkages in glycoconjugates examined to date (Kornfeld and Kornfeld, 1976).

The analysis of the linkage of newly synthesized products of glycosyltransferase reactions has posed considerable problems to investigators. If large amounts of the product can be synthesized, then nuclear magnetic resonance spectroscopy can be applied, to establish with certainty the linkage. Exhaustive methylation (permethylation) is

also valuable, but preparing the necessary standards for chromatographic analysis, which are not commercially available, is time-consuming. Periodate oxidation, followed by borohydride reduction and mild acid hydrolysis (the Smith degradation), can be difficult to interpret when oligosaccharides are used, since a number of oxidation products can result. If only small quantities (nanomoles to micromoles) of the synthesized product are available, then conventional techniques cannot be applied to analysis of the product linkage.

Some of the difficulties associated with the latter two chemical procedures can be overcome if the acceptor monosaccharide contains a radioactive label. In this case, the sensitivity of the permethylation and periodate oxidation procedures can be considerably increased. The selective introduction of ^{3}H to the sixth carbon of terminal galactosides on desialyzed α_1 acid glycoprotein has been used by Van den Eijnden et al. (1977) to determine that a sialyltransferase from rat liver adds NAN to galactose in an $\alpha 2 \rightarrow 6$ linkage. Permethylation analysis of their sialyltransferase product revealed the presence of ^{3}H-labelled 2,3,4-trimethylgalactose, indicating that NAN was linked to galactose at the 6 position. Since only tritiated products need to be examined, the procedure provides a very selective and sensitive way of examining linkages to galactose. The only drawback to this procedure is that methylated galactose standards are not available commercially, and must be synthesized, limiting, as a consequence, the applicability of this method. If such a product were treated with periodate, and subsequently reduced with borohydride (Goldstein et al., 1965), an examination of the ^{3}H-labelled acid hydrolysis products would provide a procedure which

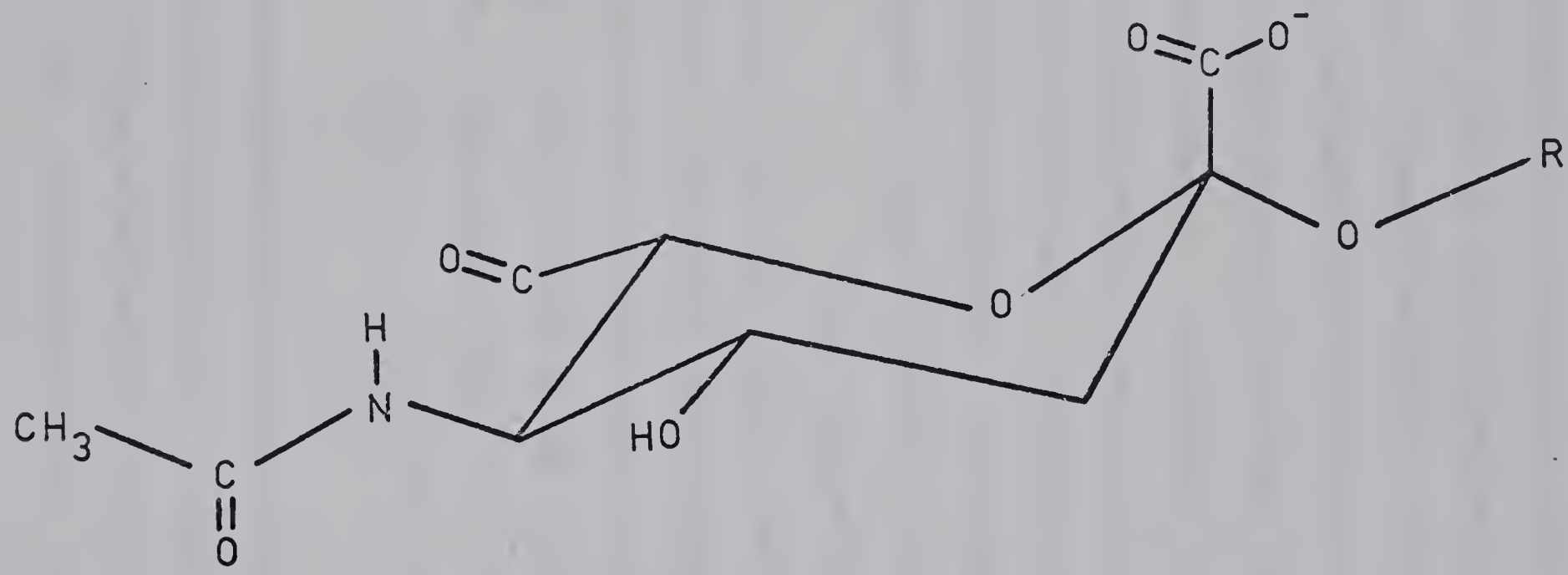
could be used without the necessity of synthesizing standards, as is now required for permethylation studies.

The linkage analysis procedure described in this report provided some interesting and unexpected results. The Smith degradation procedure worked as predicted for the galactosides which contained no sialic acid attached to them. The expected product, ^{3}H-glycerol, represented 99.9% of the ^{3}H-labelled material in the product. This would indicate that during the acid hydrolysis step, glycerol was hydrolyzed from the acetal linked to glcNAc, and did not undergo any further alteration in the acidic solution.

When sialic acid was linked to the galactose acceptor, it was expected that tritiated products would be: for a 2→2 linkage, glycerol; for a 2→3 linkage, D-galactose; for a 2→4 linkage, D-threitol; and for a 2→6 linkage, glycerol. However, when NAN was enzymatically linked to the acceptor, at least two peaks appeared on chromatograms after the Smith degradation which did not correspond to any of the expected standards. Although no information on the nature of the linkage of NAN to galactose could be obtained in the present work, modifications of the procedure used in this report may allow the linkage to be characterized.

Periodate oxidation of α-sialosides has been examined by other investigators. Oxidation, when carried out at pH 4.5, at 0° C in the dark, cleaves between carbons 7 and 8, and between carbons 8 and 9 of sialic acid, producing one mole of formic acid and one mole of formaldehyde (Tuppy and Gottschalk, 1972). The remainder of the sialic acid molecule stays intact; an aldehyde function is present on carbon 7 (Figure 23). Reduction of the oxidized NAN with sodium borohydride

Figure 23. Product of the treatment of α-sialosides with periodate at pH 4.5, 0°C, for 10 hours.


O
C
O⁻
R
O
O
C
O
H
N
CH3
C
O
HO

produces a C-7 alcohol group. In glycoproteins, other carbohydrates are also oxidized.

In this report, it was found that the ratio of $^{3}H/^{14}C$ was nearly identical before and after the oxidation/reduction procedure. Since NAN was labelled at C-4 with ^{14}C, this result was not unexpected, as this carbon should remain attached to the glycoprotein after oxidation and reduction.

After the acid hydrolysis step, and subsequent passage of the mixture through cation and anion exchange columns, no ^{14}C could be detected in the eluant. NAN is known to undergo a series of reactions in strong acid, which involves enolization and dehydration to form furan derivatives, and polymerization to form humin (Gottschalk, 1962). The carboxyl group, however, remains, and these products were probably retained on the anion exchange column.

As mentioned, the unexpected products represented by minor peaks A, C, and D in Figure 19 make it difficult to assess the type of linkage of the NAN attached to galactose. The amount of tritium present under the minor peaks A, C, and D was approximately 2.5-3.0% of the total amount present on the chromatogram, the remainder being present in the large glycerol peak. The sialyltransferase added sialic acid only to 2% of the terminal galactose residues present on desialyzed α_1 acid glycoprotein (4.73 nmoles of NAN was linked to 280 nmoles of terminal galactosides). It is of interest that the products in the minor peaks, derived from the original galactose molecule represent about 2.5-3.0% of the original galactose; this is roughly the same amount of galactose (2%) to which NAN was linked. This correlation, however, is rather

crude, and more accurate studies could be performed if NAN had been linked to a greater percentage of the terminal galactosides (such as 20-100%).

The appearance of tritium-labelled material which migrated at a faster rate than glycerol in the "experimental" sample indicates that the Smith degradation did not proceed as expected when sialosides were linked to the galactose. This result was peculiar, since the procedure worked when sialic acid was not attached to galactosides. The results indicate that glycerol remains unaltered during the procedure, since ^{3}H-glycerol was obtained as the sole product in controls in virtually quantitative yield. Glycerol, then, would not appear to undergo any reactions during the strong acid hydrolysis and subsequent work-up procedure. Threitol, another polyhydroxyl compound, would be expected to behave similarly to glycerol, and probably should not have undergone subsequent alteration. However, galactose, if present after hydrolysis, may have reacted through its aldehyde group, leading to other possible products.

The acid hydrolysis of glycoproteins often results in a reaction termed the "Maillard" or "browning" reaction (discussed by Gottschalk, 1972). Glycoproteins hydrolyzed with strong acids at 100° C often turn the solution brown, and soluble and insoluble humin appears in the mixture. The reaction is complex, but involves a reaction between amino acids in the hydrolysate with acid degradation products of sugars, in particular, with 5-hydroxymethyl-2-furaldehyde. Normally, though, in 2 N H_2SO_4, sugars are not degraded to fural derivatives, and more drastic conditions are generally required. In fact, for compositional

analyses of constituent sugars of glycoproteins, a treatment with 2 N H_2SO_4 at 100° C is commonly used for up to 6 hours (Spiro, 1966). The reason for browning in the hydrolysate in this experiment is not known, but controls were also noted to brown; this appeared to have little effect on the glycerol that was released. Hydrolysis was performed using 2 N H_2SO_4 so that any galactose that remained after periodate oxidation/borohydride reduction would be cleaved from its linkage to glcNAc. It is known that fragments of sugars produced by periodate oxidation are released from their acetal linkage under less strenuous acidic conditions (80° C, 1 hr). This is because periodate oxidation/borohydride reduction results in fragments which are linked by an acyclic acetal linkage, which is much more sensitive to acid hydrolysis than the acetal linkage of glycosides (Goldstein et al., 1965). H_2SO_4 in a concentration of 2 N at 100° C should have hydrolyzed both the acyclic acetals and the glycosides present in the mixture, releasing alcohols, aldehydes, and free sugars into solution. A reaction between the carbonyl group of galactose, and amino groups present in the mixture may have occurred. It is also possible that some unknown constituent of the plasma membranes interacted with the galactose as well.

Although the procedure of linkage analysis did not yield an answer to the type of linkage catalyzed by the sialyltransferase, the procedure still looks promising, with perhaps the following alterations:

A. Acid hydrolysis should be carried out under milder conditions (80° C, 1 hr) to release the degradation products linked through acyclic acetals. Then, after neutralization of the mixture, galactosides, if present, might be cleaved by β-galactosidase.

B. NAN should be linked enzymatically to a higher percentage of the galactoside termini present (at least as high as 10%).

Periodate oxidation has been used successfully in the past to determine sialoside linkages to galactose in glycoproteins. Results appear definite only when the linkage is α2 →3 to galactose. In this case, galactose is not oxidized by periodate, and is present in the same amounts before and after the degradation procedure (Spiro, 1964; Rearick et al., 1979). When sialic acid is linked to other positions of this sugar, however, polyalcohols are produced, which become difficult to separate from other polyalcohols that are produced due to the degradation of other sugars present in the glycoprotein. Interpretation of these results becomes difficult when different sugars are degraded to produce the same polyalcohol. The introduction of radioactive labels into specific sugars may allow this procedure to be utilized to a greater extent in the future.

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